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TITLE: POTENTIAL GENETIC ENGINEERING OF THIOBACILLUS FERROOXIDANS TO OPTIMIZE THE LEACHING OF MINERAL VALUES

FINAL REPORT

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SUMMARY

The report considered the potential for improving microbial leaching of metal sulfides by Thiobacillus ferrooxidans through genetic engineering. The report was based on a computer search of the literature dating back to 1972 and subsequent secondary references. Three areas for genetic engineering were targeted: tolerance to heavy metals; tolerance to low temperature and increased rates of metal sulfide oxidation.

In the long run there appear to be opportunities for improvement of microbial leaching through genetic engineering. However, the review of the literature suggests that at this time neither the genetic techniques nor the biochemical background is available to indicate potential genes which might be cloned to improve leaching properties of T. ferrooxidans. Thus the immediate priority for T. ferrooxidans research should be to better understand the physiology and genetics of the organism. More specific research goals are summarized below. The results from each of these areas will be interrelated so that success in one will further research in others. As none of these areas can be ignored it was deemed counter productive to attempt to list them in order of priority.

In pursuing these studies we strongly recommend that the research be carried out on strains clearly devoid of acidophilic contaminants. Ensuring purity requires sophisticated methods of identification of Thiobacillus. It

would be particularly useful if most research concentrated on a single strain. We would favor the ATCC strain used by Holmes.

The specific considerations concerning research are as follows:

1. Several outstanding problems prohibit the identification of the rate limiting step in the oxidation of metal sulfide. In particular the role and organization of the outer membrane; the enzymology of sulfide oxidation and the mechanism for establishing and maintaining the membrane potential need to be determined.
2. The adherence properties of T. ferrooxidans, particularly those which lead to specificity in adherence, deserve close attention. In addition, the question of whether adherence is required for maximum rate of oxidation needs answering.
3. The potential exists to introduce resistance to heavy metals into T. ferrooxidans since other bacteria have appropriate genes. The key would be to resolve the problems of genetic engineering described below.
4. In contrast, the lack of information on the basis of psychrophily in any organism indicates that the likelihood of creating psychrophilic strains of T. ferrooxidans through genetic engineering is remote. Traditional selection procedures will likely prove more successful.

5. Considerable effort needs to be applied towards the following problems of the molecular genetics of T. ferrooxidans:
   a. Techniques for transferring genetic material into T. ferrooxidans.
   b. Techniques for generation and selection of mutants.
   c. The structure and expression of T. ferrooxidans genes in both natural and foreign hosts.
   d. The structure and stability of the T. ferrooxidans genome, including additional analysis of plasmids which replicate in T. ferrooxidans.
6. Finally, while outside the scope of this report, we feel that a thorough study of the possible environmental risks associated with creating an "improved" T. ferrooxidans should be carried out.

RESUME

Le rapport visait à évaluer l'utilisation potentiel des techniques du génie génétique en vue de permettre un meilleur lessivage des métaux sulfurés par Thiobacillus ferrooxidans. Le rapport était basé sur une revue de la littérature (1972 à nos jours) effectuée par informatique, ainsi que sur certaines autres références secondaires. Les trois aspects visés par le génie génétique concernaient: la tolérance aux métaux lourds, la tolérance aux basses températures, de même que les taux accrus d'oxydation des métaux sulfurés.

Il apparaît évident que par l'intermédiaire du génie génétique, des opportunités s'offrent pour améliorer le lessivage microbien des sols. Cependant, la revue bibliographique démontre qu' à ce jour, les techniques génétiques et les données biochimiques fondamentales ne sont pas suffisamment connues pour indiquer les gènes potentiels qui pourraient être clonés dans le but d'augmenter les propriétés de lessivage de T. ferrooxidans. De ce fait les priorités immédiates de la recherche sur T. ferrooxidans devraient être axées sur une meilleure compréhension de la physiologie et de la génétique de cet organisme. Des objectifs de recherches plus spécifiques sont énumérés ci-dessous. Les résultats pour chacun de ces aspects sont intimement reliés; le succès de l'un favorisera le développement et l'avancement des autres. Puisqu'aucun de ces aspects ne mérite d'être ignoré, il n'apparaît donc pas

nécessaire de tenter de les classer par ordre de priorité.

Pour la poursuite de ces études, il s'avère indispensable que la recherche soit effectuée sur des souches clairement exemptes de contaminants acidophiles. Des méthodes complexes d'identification de Thiobacillus sont ainsi requises pour s'assurer de la purete. Il serait donc particulièrement utile et souhaitable d'axer les études sur une seule souche'nous favoriserions la souche type de l'ATCC déjà utilisée par Holmes.

Les considerations specifiques concernant le domaine de recherche sont comme suit:

1. Divers problemes majeurs empêchent l'identification de l'étape limitante dans l'oxydation des métaux sulfurés. Le rôle et l'organisation de la membrane externe, l'enzymologie de l'oxydation sulfureé ainsi que le mecanisme pour l'établissement et le maintien du potentiel membranaire devraient entre autres être déterminés.
2. Les propriétés d'adhérence de T. ferrooxidans, particulièrement celles reliées à la spécificité de l'attachement, méritent une attention particulière. De plus, l'existence d'une corrélation entre l'adhérence et un taux maximum d'oxydation devrait être évaluée.
3. La possibilité d'introduire une résistance aux métaux lourds chez T. ferrooxidans est existante puisque d'autres espèces bactériennes possèdent les gènes appropriés. Pour ce faire, il serait toutefois nécessaire de resoudre d'abord les problèmes de la

génétique moléculaire cités un peu plus loin.

4. Au contraire, le manque évident d'information sur le fondement de la psychrophilie indique que la création par manipulations génétiques de souches psychrophiles de T. ferrooxidans est peu probable.

5. Des efforts considérables devront être mis de l'avant pour pallier les problèmes suivants de la génétique moléculaire de T. ferrooxidans:

   a. Les techniques pour le transfert de matériel génétique à l'intérieur de T. ferrooxidans.

   b. Les techniques pour la production et la sélection de mutants.

   c. Une compréhension de la structure et de l'expression des gènes de T. ferrooxidans, chez l'hôte naturel et chez l'hôte étranger.

   d. Une compréhension de la structure et de la stabilité du génome de T. ferrooxidans, incluant des analyses additionnelles de plasmides se répliquant chez T. ferrooxidans.

6. Finalement, bien qu'en dehors du champ immédiat de ce rapport, nous croyons qu'une étude approfondie démontrant les risques possibles pour l'environnement, associés à la création d'une souche "améliorée" de T. ferrooxidans, devrait être envisagée.

INTRODUCTION

A. SCOPE OF THE REPORT

This report was completed in accordance with the terms of DSS contract Serial No. OSQ85-00230. The work statement for the contract called for a review of literature on: (1) the genome of T. ferrooxidans; (2) intracellular plasmids of T. ferrooxidans; (3) the enzymology of metal sulfide oxidation; and (4) effects of temperature and heavy metals on growth and activity. Having completed the review, a rational genetic engineering program to improve leaching using T. ferrooxidans was to be devised.

The objective of the report was to consider the potential for improving microbial leaching of metal sulfides by T. ferrooxidans through genetic engineering of the organism. Three areas for modification of T. ferrooxidans were targeted: (1) tolerance to heavy metals; (2) tolerance to low temperatures; and (3) metal sulfide oxidation capacity. For each area, we looked for critical characteristics whose alteration might improve the capabilities of T. ferrooxidans. In the area of metal sulfide oxidation, two factors which are under biological control were investigated: (1) Mechanisms of attachment of the bacteria to the substrate are of importance since the substrate is insoluble. (2) The enzymology of sulfide oxidation was reviewed to ask whether there are steps which clearly limit the oxidation rate and which might be readily manipulated. Finally, the current state of the molecular genetics of T. ferrooxidans was reviewed since a thorough knowledge of this area is essential for genetic engineering.

B. METHODS

The study was initiated with a computer search of the literature based on the identification of all reports concerned with acidophiles. The search yielded over 1400 primary references dating from 1972. Relevant references were collected and read and a list of secondary references was identified. A series of literature review seminars were held in which materials were presented and analyzed. When there was common agreement on the current state of a topic one of the reviewers was assigned to write a summary of the material.

The references reviewed in the report are listed in the bibliography.

C. BACKGROUND

In the preparation of the report a number of axioms emerged. These formed the starting point for our review, and they are presented below.

1. Significance of T. ferrooxidans

Because of the importance of T. ferrooxidans in microbial leaching, it seemed reasonable that a review of the potential for genetic engineering in this organism be undertaken. It is well established that the organism is capable of solubilizing metal sulfides and oxidizing reduced forms as a means of generating ATP. There is widespread appreciation that T. ferrooxidans is not the only organism found in leaching conditions and the idea that other organisms contribute to leaching either directly or by aiding the activity of T. ferrooxidans has been demonstrated. Furthermore, there are a number of organisms which can facilitate the oxidation of metal sulfides.

On the whole, our review suggested that in the long run there are opportunities for the improvement of microbial leaching through the genetic engineering of *T. ferrooxidans*. On the other hand, before genetic engineering of *T. ferrooxidans* can be successful, a significant body of research into the physiology and genetics of this organism will have to be carried out.

2. Classification of *Thiobacillus* Species

The Thiobacilli are defined as Gram negative, non-spore forming, aerobic, motile, rod-shaped bacteria which derive energy from oxidation of reduced or partially reduced sulfur compounds. They share the property of being able to grow between pH 2 and pH 5 and several species are able to grow at pH values below 2. There are a large number of species types which fit into the general classification of *Thiobacillus*. Many of the *Thiobacillus* species appear only distantly related to *T. ferrooxidans*. In what seems to be an important study, Lane *et al.* (1985) classified the *Thiobacilli* using 5S RNA homology (see page 28). These studies correlated well with other information showing that *T. ferrooxidans* is rather closely related to *T. thiooxidans*, but is very distantly related to most other *Thiobacillus* species. In particular, non-acidophilic and heterotrophic *Thiobacilli* are likely to be fundamentally different from *T. ferrooxidans*. This genetic separation implies that caution must be applied when using non-acidophilic or heterotrophic *Thiobacillus* species as models for *T. ferrooxidans*. On the other hand, it should be reccgnized that these other *Thiobacillus* species may be useful under some leaching conditions.

3. General Considerations of the Microbiology of Leaching Operations

Microbes are used for leaching under a diversity of conditions which reflect the differences between leach operations. For example the type of ore introduced into the operation may vary from one site to another and possibly at one site from day to day. The changes in the characteristics of the ore could introduce selective pressures on the microbial populations. The characteristics of the leaching operation itself may necessitate different microbial characteristics. For example, microbial adherence may be less important in stirred tank reactors than in heap leaching. Furthermore, the question of whether rate or final yield is the most important parameter may vary depending on the leach operation. As a result, the object of genetic engineering of T. ferrooxidans may be to create an organism which can rapidly adapt to changing conditions rather than to mold the organism to a presently identified situation.

In designing an "improved" T. ferrooxidans species, the key question to resolve is what features limit bioleaching. In reviewing the literature on metabolism and the enzymology of metal sulfide oxidation and the inhibitory effects of temperature and toxic metals, we attempted to discern rate limiting steps which might be targets for manipulation. Ultimately thermodynamic parameters of the chemical reactions involved in generating ATP from metal sulfide oxidation will limit the rate of bioleaching. Thus it is expected that there is an ultimate limit to the rate. We do not at present know how close to that limit T. ferrooxidans operates. In other examples of the use of microbes for biochemical processes, the organisms are believed to

be very close to the theoretical limits, e.g. the production of lysine from carbohydrates. Furthermore, the metabolic pathways of T. ferrooxidans are likely to be highly specialized and therefore tightly coupled. Thus increasing the intracellular concentration of one protein may do little to increase substrate turnover since another product may become rate limiting. Such is believed to be the case for the slowly growing nitrifying bacteria. As a result, a considerable amount of information on the metabolism of T. ferrooxidans is a prerequisite for successful genetic engineering.

4. Genetic Engineering

Genetic engineering is the purposeful manipulation of genetic structure of an organism. The most commonly thought of method used is to isolate a gene by molecular cloning and then to reintroduce that gene so that its activity is altered from the wild type state. The basis of genetic engineering is thus molecular cloning (see Figure 1). The process of cloning involves a series of four steps. The first three of the steps are largely biochemical and usually are essentially the same for all recombinant DNA experiments. The final step is basically genetic and thus must refer specifically to the system being manipulated.

The initial step in cloning is to isolate the genetic material (the DNA) from the organism whose gene is to be cloned. This is a straightforward biochemical procedure. Within the DNA are segments which contain the genetic information for all of the activities of the organism (the "genes"). The DNA of the organism is then treated with one of an unusual class of enzymes:

restriction endonucleases. These enzymes are unique in that each type of restriction endonuclease recognizes a particular sequence of nucleotides in the DNA and will cleave the DNA at that sequence. For example, one type of restriction endonuclease recognizes the sequence of six nucleotides GAATTC which occurs roughly once in every 4096 nucleotides. The recognition sites occur randomly in any given DNA, but because they are duplicated with great fidelity during cell division, they are a recognizeable characteristic of the DNA. The key importance of these enzymes are that they cut a genome (which is roughly 100 million nucleotides long in the average bacterium) into defined pieces each ending in the same sequence. Certain of the restriction endonucleases yield what are termed staggered cuts, which although they are not necessary, make the next step somewhat easier.

The next step in molecular cloning is to attach the DNA from the target organism to a small DNA molecule called a cloning vehicle. The cloning vehicle must have three characteristics. First, it has a restriction endonuclease site whose ends are compatible with those used to cleave the DNA containing the gene to be cloned. Secondly, the cloning vehicle must have genes which are easily recognized. Ideally the vector will have two genes, one of which will contain the restriction endonuclease site (the cloning site). Thirdly, the cloning vehicle must have an origin of replication, that is a DNA sequence which, when inside a host organism, will interact with the enzymes of that organism and lead to replication of the cloning vehicle. Anything linked to the cloning vehicle will also be replicated. By attaching the fragments of the DNA to the cloning vehicle and introducing the recombinant DNA into a host organism the fragments of DNA can be maintained

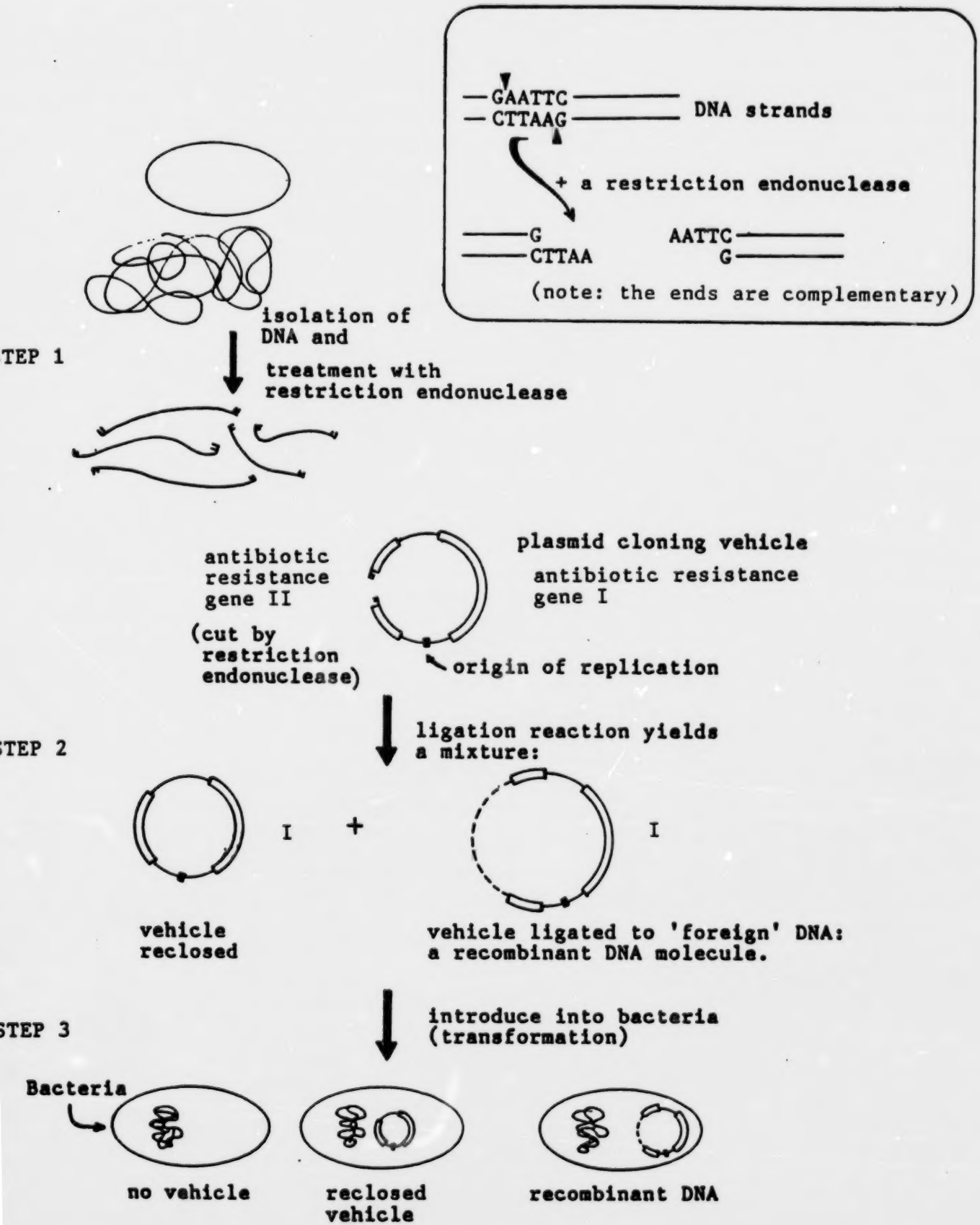


Figure 1. Basics of genetic engineering

STEP 4 Selection of bacteria carrying cloned gene.

Bacteria from the transformation step are grown in media containing antibiotic I; only cells carrying the cloning vehicle survive. Survivors are plated on media containing antibiotic I, grown into colonies and cells from each colony are tested on plates containing antibiotic II.

antibiotic I antibiotic II

In recombinant DNAs, the "foreign" DNA interupts the gene for resistance to antibiotic II, destroying its function. Therefore cells which grow in the presence of antibiotic I, but not antibiotic II carry recombinant DNA.

A collection of cells carrying recombinant DNAs is assembled from the test above. Those cells are then tested using an assay which is specific for the gene to be cloned.

and produced in large amounts by growing the bacteria. The linking of the DNA to be cloned with the cloning vehicle is carried out by a purified enzyme (DNA ligase).

In the third step of the cloning procedure, the products of the ligation reaction are then added to bacteria which are in a particular state where they will take up DNA from the medium. This latter state (competence) is the natural part of the life cycle of some bacteria, but must be induced by chemical treatments in other bacteria.

At the end of the third step of the cloning procedure, one has a culture of bacteria many of which carry a cloning vehicle which may, or may not, contain a fragment of foreign DNA. In addition, only a small fraction of the bacteria (and their direct descendants) will carry the particular gene which was to be cloned. Thus the final step in the procedure is to select the bacteria (and their descendants) which carry the gene of interest by a series of genetic tests.

In the ideal case, the cloning vehicle genes are used in the initial selection process. For example, two of these genes might code for resistance to antibiotics. After transformation with the ligation mix, the host bacteria are tested for resistance to the antibiotic whose gene did not carry the restriction endonuclease site by adding the antibiotic to the growth medium. Those bacteria which grow are resistant and must carry the cloning vehicle. Progeny of these bacteria are then tested for resistance to the second antibiotic. Since insertion of the cloned DNA will disrupt the antibiotic resistance gene and thus inactivate the gene product, bacteria which carry a cloning vehicle with inserted DNA will be sensitive to the second antibiotic.

By testing the cells in culture for the pattern of antibiotic resistance, one can obtain a collection of bacteria which have a wide variety of pieces of DNA from the organism whose gene was the target of the cloning. This collection of bacteria represents a "gene bank" and assuming that it is complete the bank will contain the gene of interest. The next step is to devise a test which will identify which member of the gene bank carries the gene of interest. The identification requires either a test of activity of the gene or a biochemical method to identify the gene product or the gene itself. Clearly these tests must be devised to suit the particular cloning situation.

Once a gene has been isolated, there are a number of ways that it can be altered to change its activity. These means include altering regulatory controls which act on the gene; changing the number of copies of the gene; changing the cellular localization of the product and finally altering the primary sequence of the gene. In general the cloning procedures use vectors and vehicles which are well characterized (such as E. coli and its plasmids). Thus a direct test for a cloned gene requires that the gene produce a product in E. coli. Transfer of the gene back to the original organism requires a vehicle which replicates in that organism.

5. Genetic Engineering of T. ferrooxidans

In the case of genetic engineering of T. ferrooxidans, the primary interest is most likely to increase expression of a gene already present or to create an organism with a new genetic trait. Mechanisms of increased tolerance to toxic metals, or increased levels of binding proteins for sulfur

6. Oxygen

O_2 is often cited as a possible limiting factor in situ. Myerson's data (1981) however found that the limiting concentration of oxygen was always less than 5% of saturation.

7. Toxic Agents

Tuttle and Dugan (1976) demonstrated inhibition of iron oxidation, sulfur oxidation and growth by a wide variety of low molecular weight organics. More specific to the mining situation was the data by Torma and Itzkovitch (1976) and Tuovinen (1978) that indicated that the organic solvents used for metal extraction and several flotation agents are equally toxic.

C. SULFUR OXIDATION

1. Enzymes

Several enzymes have been isolated from different Thiobacillus species each capable of oxidizing different sulfur compounds. Evidence is accumulating that they are probably located in the periplasm as shown in Figure 3 (Hooper and DiSpirito, 1985).

The ability of all Thiobacillus sp. to utilize sulfur compounds makes the previously mentioned problem of the purity and taxonomy of T. ferrooxidans particularly acute when considering sulfur oxidation. In addition, T. ferrooxidans has a history of fastidious growth requirements. Because of this most of the data on sulfur oxidation has been obtained from related species. Kuenen and Beudeker (1982) categorized the thiobacilli into the following groups based on their metabolism (Table 1).

Perhaps a more useful idea of the relatedness of T. ferrooxidans to other Thiobacilli mentioned in the sulfur oxidation literature is provided by 5S rRNA homologies (Lane et al., 1985), Figure 4. This study indicates very close genetic similarity between T. ferrooxidans and T. thiooxidans (5S rRNA homology, 0.98). The other Thiobacilli which are clustered in Group II, subgroup a with T. ferrooxidans are T. concretivorus and T. thioparus. All these species are obligate chemolithotrophs with a ubiquinone of 8 isoprenoid units.

All the enzymes shown in Figure 3 have been identified in T. ferrooxidans (Tuovinen et al., 1976) except sulfite reductase and adenine phosphosulfate (APS) reductase. Siroheme sulfite reductase was purified in

might be examples. The fact that the ultimate aim will be to express proteins in T. ferrooxidans leads to certain requirements for success.

a. The genetic signals for gene expression at both the RNA and protein levels must be understood. These signals are crucial to isolate and manipulate genes. Information from related organisms can be used, as long as it is certain that the signals are compatible. This already has been demonstrated in T. ferrooxidans. The question can be more subtle than might be expected, however. For example, most mRNAs from Gram positive bacteria are easily translated by cell extracts of Gram negative cells. On the other hand most mRNAs from Gram negative cells are not translated in Gram positive cells. The block appears to be in a stringent requirements for nucleotide sequences at the 5' end of the mRNAs.

b. A means to introduce genes into T. ferrooxidans must be available. There are a wide range of options for many bacteria: plasmid directed transfer (conjugation), lysogenic bacteriophage, transformation, protoplast fusion and electroporation. Ultimately it is not critical which method is available, although each method has some limitations and will therefore limit the capacity of the research. Demonstration of genetic exchange, in turn, requires defined

genetic variants (mutants) as genetic markers. Thus a prerequisite for a means of genetic exchange is to define a test gene which is certain to be expressed in T. ferrooxidans and which, when expressed, will be recognized.

c. A method for stably maintaining genes in T. ferrooxidans must be available. In general genes can be maintained either on plasmids or by integration into the cellular chromosome. Integration may take place after entry of the DNA through transformation, conjugation, or infection with a bacteriophage carrying the gene. Integrative mechanisms may be more stable and thus reliable; this has been found to be the case in other bacteria, notably Bacillus. Plasmids offer the ability to have high gene dosage through copy number. For some applications, the high copy number can be very useful (toxic metal resistance, for example). However, in other cases, over production of a cloned gene product can be toxic.

d. Finally a means to select the genes to be cloned is required. While there are a number of ways to select genes without using gene activity (assay by antibodies, for example) the ability to reintroduce the gene into T. ferrooxidans means that a method for selecting cells which have received the cloned gene is essential.

6. Control of Microbial Populations

A key aspect of fermentation processes is the monitoring and control of the microbial population. In the case of a process based on an artificially selected strain, the stability of the strain during the fermentation and the ability of the strain to compete with potential contaminants are particularly important. The latter considerations are particularly significant in microbial leaching since there is little data on the survivability of laboratory strains in leaching situations and the substrate is not likely to be sterile. The technology to be able to control the microbial population is based on means for identifying particular strains of an organism. This technology must be a primary concern in developing improved microbial leaching.

7. Environmental Risks

As a final introductory note, the potential for environmental risk posed by an improved T. ferrooxidans species should be considered. It is well known that T. ferrooxidans is the source of acid water problems in a number of areas around the world, including Canada. Given the scale and method of bioleaching, it is highly improbable that genetically improved strains would be contained. Indeed it is possible that litigation would prevent the field use of genetically engineered T. ferrooxidans even if one were created. While we considered this problem outside the scope of our report, it is a sufficiently important problem that we felt obliged to comment on it.

METABOLISM AND GROWTH

A. INTRODUCTION

The obligately chemoautotrophic mode of metabolism of T. ferrooxidans is unusual amongst the Thiobacillus. T. ferrooxidans is able to derive energy not only from the oxidation of reduced sulfur compounds (as can all thiobacilli) but also from the oxidation of ferrous to ferric iron. The bacterium is able to couple the energy from these oxidations to the reduction of NAD^+, fixation of CO_2 and hence the construction of cell biomass.

In the field of mineral leaching, bacteria are known to be involved in two of the three mechanisms of metal dissolution. These mechanisms are (1) direct attack and oxidation of ferrous iron (Beck and Brown, 1968), (2) indirect attack by ferric iron on the sulfide moiety (Lundgren and Silver, 1980), and (3) galvanic interaction (Murr and Mehta, 1983). It is the first two which are believed to be influenced by the activities of T. ferrooxidans.

Accordingly, this section will concentrate on the pathways of sulfur and iron oxidation in T. ferrooxidans and attempt to identify factors important to the control of these pathways and the bacterium. The material includes:

1. iron oxidation in T. ferrooxidans
2. sulfur oxidation in T. ferrooxidans
3. CO_2 fixation in T. ferrooxidans
4. psychrophily in T. ferrooxidans
5. mineral leaching and T. ferrooxidans

6. possible approaches to improved leaching by T. ferrooxidans.

In the final section the feasibility of manipulating factors through cloning will be discussed.

B. IRON OXIDATION

1. Electron Transport Components

The observation that respiratory poisons (Duncan et al., 1967) and organics (Tuttle and Dugan, 1976) can selectively inhibit iron oxidation with no effect on sulfur oxidation has provided long standing evidence that the iron oxidase electron carriers are distinct. Furthermore, they are reported to be constitutive (Margalith et al., 1966). However a recent abstract (Mjoli and Kulpa, 1986) suggests rusticyanin is induced by iron. The chain is believed to be very short consisting of three electron carriers; rusticyanin, cytochrome c (cyt.c) and cytochrome a_1 (cyt. a_1). Bodo and Lundgren (1974) indicated that iron oxidation is associated with the cell envelope. More recent sophisticated investigations with paramagnetic probes (Ingledew, 1982) suggests that rusticyanin and cyt. c are periplasmic while cyt. a_1 is located in the cell membrane. The extra-cytoplasmic oxidation can also be inferred from the fact the iron would rapidly auto-oxidise at cytoplasmic pH values (pH 6.5) and that Fe^{3+} formed at this pH would be insoluble.

Figure 2 depicts the most likely conformation of the electron transport chain based on present data.

When grown on acidic $FeSO_4$ media, electrode potentials of the growth media can reach values of >750mV when the concentration of Fe^{2+} in the medium is less than 5mM. This ferrous scavenging is attributed to either a binding

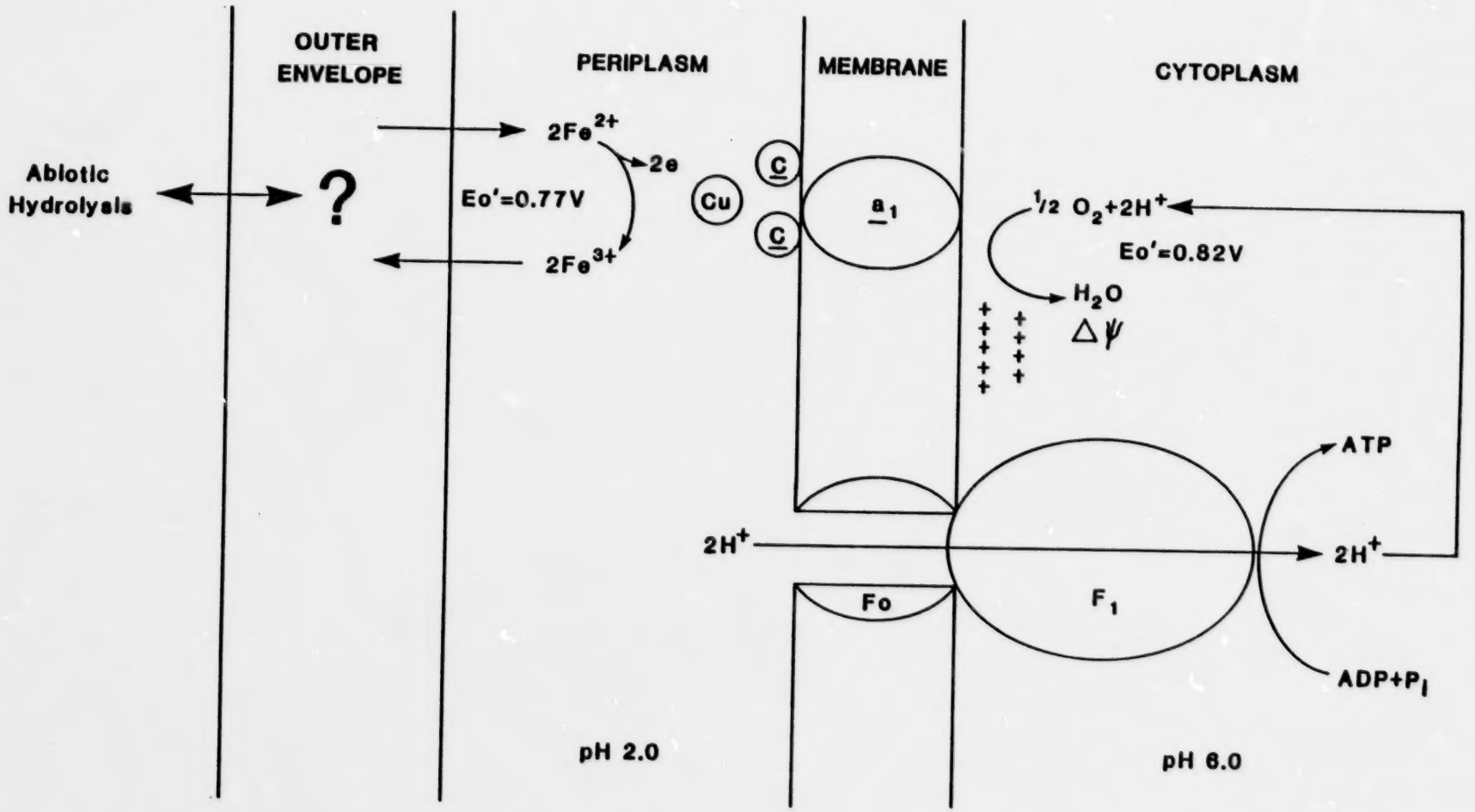


FIG. 2 CURRENT SCHEMES OF IRON OXIDATION

(Modified from Cox & Brand 84, Ingledew 82, Hooper & DiSpirito 85)

protein which interacts with rusticyanin or to rusticyanin itself. The Km for the reduction of rusticyanin is 0.5mM (Cox and Brand, 1984). Even though rusticyanin possesses the necessary high substrate affinity for such scavenging, the small change in the free energy between the Fe^{2+}/Fe^{3+} and the 0.5 O_2/H_2O couples at physiological pH does not release sufficient energy for ATP production (Tuovinen and Kelly, 1973). Silver (1978) therefore postulated the need for an organic to complex with rusticyanin to lower the E_0 sufficiently to produce the necessary free energy for phosphorylation.

Coupling of phosphorylation and iron oxidation in T. ferrooxidans is essentially chemiosmotic (Ingledew, 1982; Ingledew and Cobley, 1980). The proton electrochemical gradient is established primarily by the Δ pH across the cytoplasmic membrane. Hooper and DiSpirito (1985) stated that the oxidation of Fe^{2+} as a substrate is unusual among simple reductants as no protons are produced from the Fe^{2+}. This explains the obligatory acidophilic nature of iron oxidation. The acidic exterior milieu is necessary to establish the electrochemical gradient.

The maintenance of the Δ pH has not yet been satisfactorily explained. There is a large passive component as uncouplers, protonophores and inhibitors do not collapse it. Respiration is known to account for < 20% of the Δ pH (Matin et al., 1982). The positive $\Delta\phi$ on the inside of the membrane restricts the influx of protons except through F_0 - F_1 channels. Under balanced growth every proton influxed to effect ATP-ase activity is reduced to H_2O via cyt. a_1 (figure 2). The establishment of this membrane potential was once ascribed to a Donnan potential but this idea is now refuted. Matin's group, working with T. acidophilus (Zychlinsky and Matin, 1983a and

b), demonstrated that the cytoplasm has unusually high buffering capacity and a membrane impermeable to ions other than protons. This is somehow believed to contribute to cation sequestering and the establishment of $\Delta\phi$.

The stoichiometry of ferrous oxidation in T. ferrooxidans consumes four mol of Fe^{2+} per mol O_2 (Cobley and Cox, 1983). This gives a theoretical H^+/P and a P/O ratios of 4 and 0.5 respectively.

2. Growth and Iron Oxidation

Numerous publications list the growth rates and substrate affinities of T. ferrooxidans strains. The most common medium is the 9K medium of Silverman and Lundgren (1959) with $FeSO_4$ as substrate, although some data are available on various mineral ores. Values for maximum specific growth rates (μ_{max}) on $FeSO_4$ range from 0.07 hr^{-1} to 1.78 hr^{-1} (Kelly and Jones, 1978, Kovalenko et al., 1982, Tomizuka et al., 1976, MacDoanld and Clark, 1970, Moshnyakova and Karavaiko, 1979). K_S values range from 0.7 - 37mM (Fe^{2+} ion). The fast rate of 1.78 hr^{-1} is from a well documented chemostat study (Kelly and Jones, 1978). The most often cited rates are in the 0.1-0.2 hr^{-1} range. Figures for mineral growth are not as plentiful. Chang and Myerson (1982) obtained μ_{max} = 0.11 hr^{-1}, K_S = 2.5mM pyrite while Roy and Mishra (1981a) obtained μ_{max} = 0.09 hr^{-1}. Gormely et al. (1975) produced a μ_{max} of at least 0.10 hr^{-1} on zinc sulfide concentrate. A main cause of variability in the figures is undoubtedly uncertainty about the taxonomy and purity of the iron oxidizing thiobacilli. Variations have been reported within strains classified as T. ferrooxidans in DNA composition (Guay et al., 1976, Martin et al., 1981), physiology (Silver, 1978) and morphology (DiSpirito et al., 1981). Harrison's lab has been most active in

investigating this anomaly. In 23 strains of T. ferrooxidans examined by DNA homology, 25% were found to be contaminated with heterotrophs (Harrison, 1982). This study eventually proposed seven homology groups of authentic T. ferrooxidans encompassing a mol GC range of 52% - 62%.

The cryptic heterotrophic contaminants in T. ferrooxidans strains is perhaps a more insidious problem. Two common contaminants have now been isolated and classified as Acidophilium cryptum (Harrison, 1981) and T. acidophilus (Arkesteyn and DeBont, 1980). Many other heterotrophs exist in apparent symbiosis with T. ferrooxidans (Harrison, 1984) and are annoyingly resistant to purification (Johnson and Kelso, 1983). Because of this problem one must be cautious in interpreting much of the T. ferrooxidans data. The exacting nature of chemotrophy on Fe^{2+} helps to dispel some doubt and it is possible to state that the pH optimum for iron oxidation is in the range pH 1.8-2.1 and temperature optimum is 28-30°C.

There is a substantial body of evidence that growth and iron oxidation are tightly coupled. Braddock et al. (1984) in a chemostat study of a T. ferrooxidans isolate found an identical threshold value of external ferrous ion (0.25mM) for growth as ferrous oxidation. DiSpirito and Tuovinen (1982) found a direct relationship between the O_2 uptake/CO_2 fixation and the oxidation of uranous and ferrous ions. Similarly Roy and Mishra (1981a) found that the log of the rate of pyrite oxidation and the rate of cell multiplication were almost identical. Tuovinen et al. (1979) found that the ferrous oxidation rate in T. ferrooxidans corresponded with the concentration of available nitrogen. Lacey and Lawson (1970) were able to accurately describe the oxidation of $FeSO_4$ by the formula:

$$\frac{ds}{dt} = \frac{\mu m\ SX}{Y\ (K + S)}$$

where t = time (h), S = concentration of Fe^{2+} (g/l), μm = maximum specific growth rate (h^{-1}), Y = mass of bacteria produced per gram iron oxidized (g/g), K = saturation const. (g Fe^{2+}/l), and X = concentration of bacteria (g/l).

Eccleston and Kelly (1978) in a study on tetrathionate and thiosulfate oxidation determined that the yield was increased on both substrates at low dilution rates by increasing the CO_2 supply. This was due to a lowering of the maintenance coefficient without any effect on true growth yield in CO_2 rich environments. It is likely that CO_2 acts in a similar manner with iron oxidation.

It is only under conditions of extreme stress that uncoupling is observed. Thus Kelly and Jones (1978) produced growth uncoupled oxidation by exhausting the CO_2 supply. Roy and Mishra (1981b) showed that toxic metals inhibited growth at lower concentrations than those which inhibited iron oxidation.

3. Sulfur Oxidation and Iron Oxidation

When grown on a metal sulfide such as pyrite, iron and sulfur are both oxidized (Beck and Brown, 1968). Duncan *et al.*, (1967) reached similar conclusions growing *T. ferrooxidans* on chalcopyrite and pyrite. They further found that the contribution to O_2 uptake from iron oxidation or sulfur oxidation depended on what substrate the cell was grown on:

Growth Substrate	Oxidizable Substrate	% O_{02} Attributable to Sulfur Oxidation	% O_{02} Attributable to Iron Oxidation
Chalcopyrite	Chalcopyrite	68-74	25-30
Chalcopyrite	Pyrite	100	
Ferrous	Chalcopyrite		89-90*
Ferrous	Chalcopyrite	68-74	25-30**
Ferrous	Pyrite	20-30	70-80

* initial rate

**final rate

This study supports an earlier report by Beck (1960) that iron grown cells oxidized sulfur compounds in the presence of $FeSO_4$ only when the Fe^{2+} was completely exhausted. The preference of some cultures for iron oxidation is puzzling in light of the theoretical calculations of the number of electrons liberated and the associated free energy. Such calculations suggest that the energy available if growing on FeS_2 from Fe^{2+} oxidation is only 4.5% of that available from sulfide oxidation (Bennett and Tributsch, 1978). A consequence of the reluctance to engage in sulfur oxidation is the deposition of S^0 on the mineral crystal which prevents further dissolution.

Mixed culture studies with T. ferrooxidans, T. thiooxidans and Leptospirillum ferrooxidans indicated that some form of cooperation was

required between L. ferroxidans and T. thioxidans before the Leptospirillum could grow on pyrite. L. ferroxidans is unique in that it can only grow on iron not sulfur (Norris and Kelly, 1978b). Comparisons between T. ferrooxidans and the above mixtures of species also suggested that iron oxidation might be the limiting pathway for T. ferrooxidans growing on pyrite.

4. pH

pH values below pH 1.5 inhibit iron oxidation (Roy and Mishra, 1981a). Above pH 3.0 the removal of Fe^{3+} is the most limiting factor due to precipitation (Lees et al., 1969). Jarosite is often a visible result of this precipitation (Keller and Murr, 1982).

5. Ferric Ions

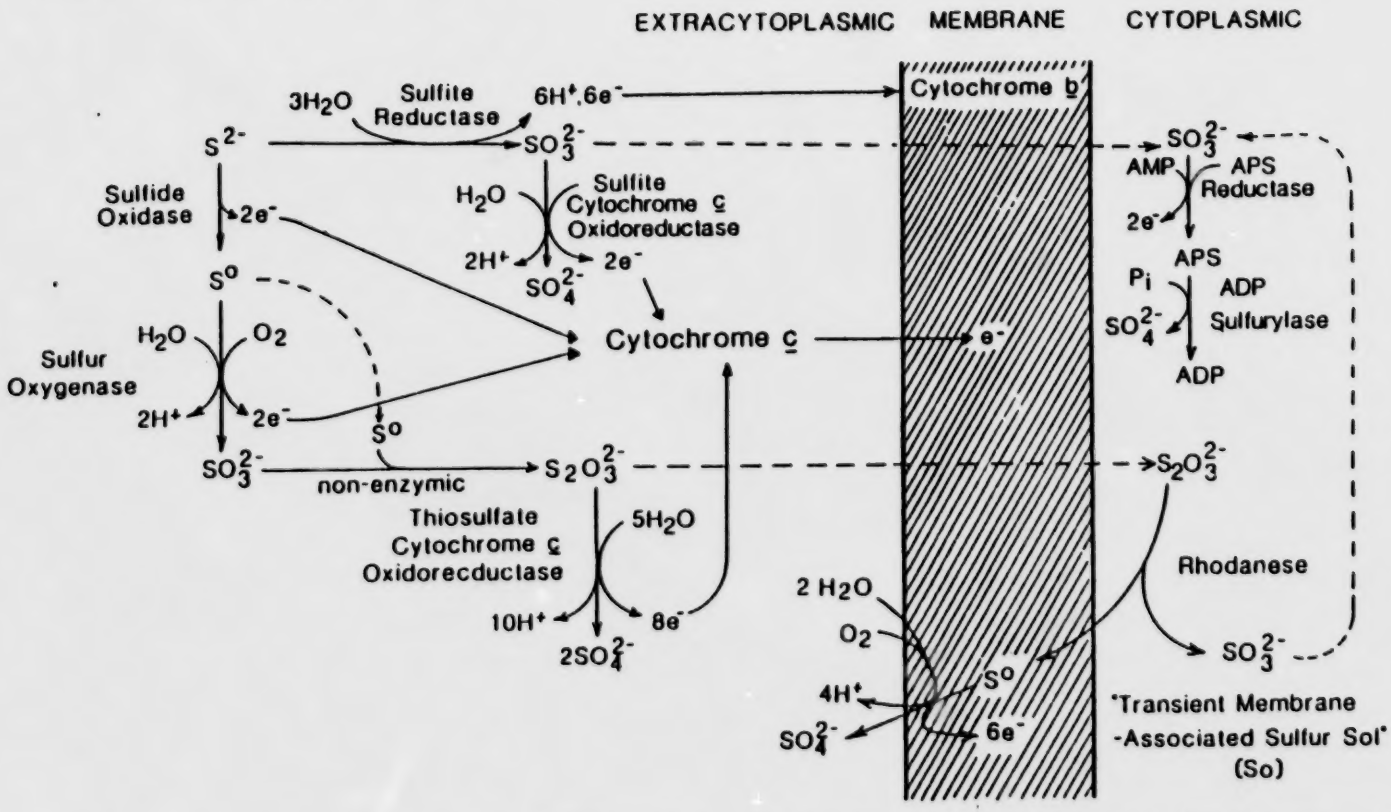
Kelly and Jones (1978) and Jones and Kelly (1983) demonstrated competition of ferrous oxidation by ferric ions. They claimed that in all batch situations the observed rate will ultimately depend on the ferrous/ferric ratio. This product inhibition is a competitive effect. Belyi and Gurevich, (1976) using an electrochemical culture vessel where the Fe^{3+} is converted to Fe^{2+}, calculated that for optimal growth of T. ferrooxidans ferric iron should be less than 13g/l with ferrous iron equal to between 3 and 5g/l.

TABLE 1

Classification of *Thiobacilli* by Metabolism

Obligate Chemolithotrophs		Facultative Chemolithotrophs		Chemolitho-heterotrophs
Aerobes	Facultative Anaerobes	Aerobes	Facultative Anaerobes	
T. neapolitanus	*T. denitrificans*	*T. intermedius*	Thiobacillus A2 (now *T. versutus*)	*T. perometabolis*
T. thiooxidans	*T. thioparus*	*T. novellus*		
T. ferrooxidans		*T. acidophilus*		
T. kabobis		*T. organoparus*		

FIGURE 3



Possible arrangement of the enzymes involved in the oxidation of reduced sulfur compounds. The scheme is a composite of the reactions catalyzed by several different species of thiobacilli.

(From Hooper and Di Spirito, 1985, with permission)

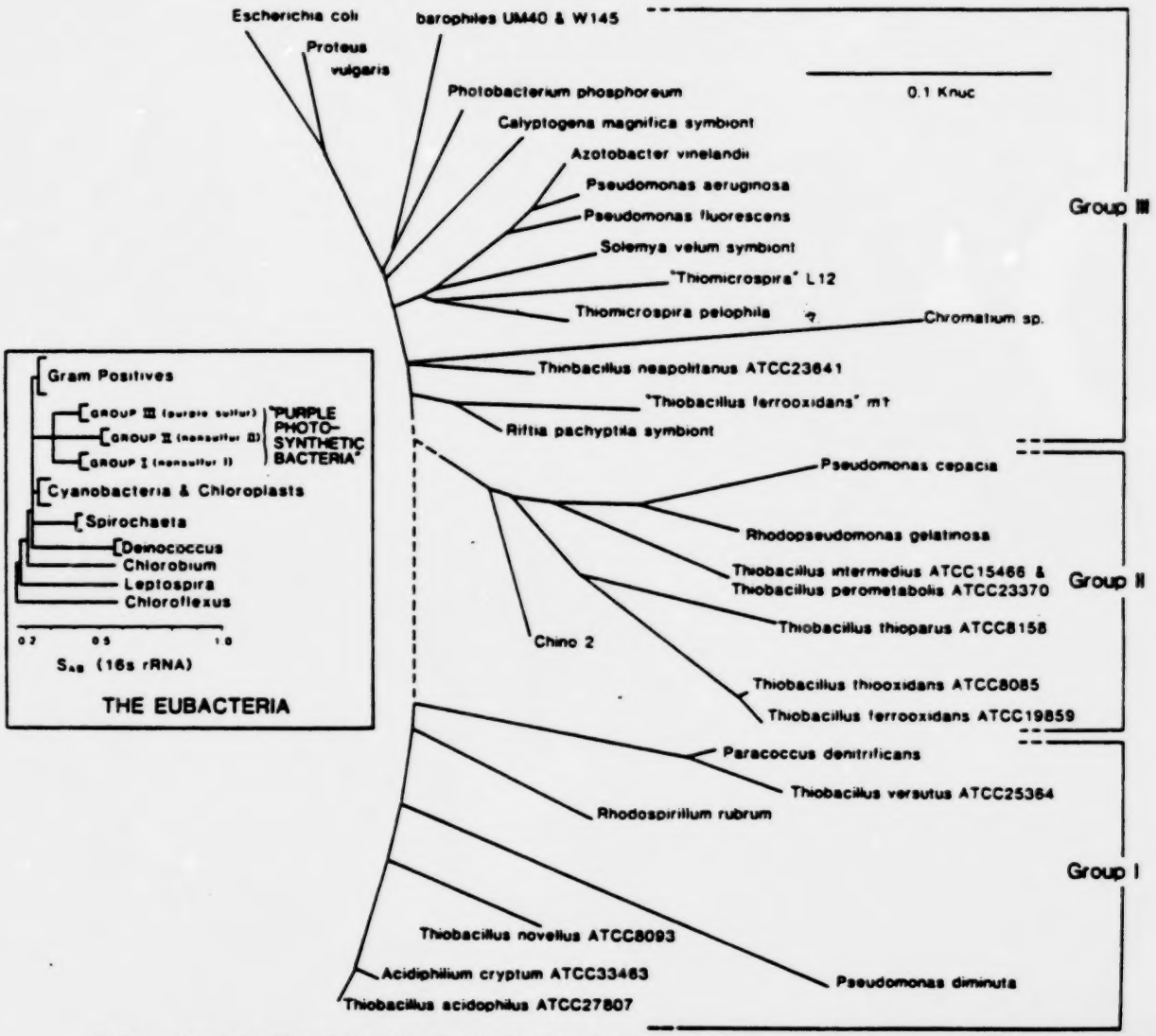
T. denitrificans (Schedel and Truper, 1979) and so the entry of reducing equivalents at cytochrome b (cyt. b) is probably a peculiarity of this species. Vestal and Lundgren (1971) found that sulfite oxidase activity in T. ferrooxidans was AMP independent, so APS reductase is probably absent. Phosphorylation through sulfite oxidation is presumably therefore all oxidative.

The data is unequivocal that T. ferrooxidans is able to grow directly on metal sulfides (Beck and Brown, 1968; Roy and Mishra, 1981a). Surprisingly a sulfide oxidase has yet to be purified from T. ferrooxidans or any other species of Thiobacillus. The initial reactions of sulfide oxidation were studied extensively in T. concretivorus (a variant of T. thiooxidans) by Moriarty and Nicholas (1969, 1970a, 1970b). Absorbance spectra investigations suggested a linear polymeric sulfur intermediate on the membrane. A copper protein was implicated in the formation of this polysulfide which caused the polymer to bind to the membrane. This was also found in T. thiooxidans and T. thioparus. Bound polysulfide always oxidized faster than free polysulfide. Saxena and Aleem (1973) found a similar polysulfide in T. neapolitanus.

Silver and Lundgren (1968) isolated sulfur oxygenase from T. ferrooxidans and found it required reduced glutathionine for activity. Its exact location has not been determined.

Thiosulfate can be oxidized by all species of Thiobacillus. Two different oxidation enzymes exist; the thiosulfate oxidizing enzyme and rhodanese. Both have been identified in T. ferrooxidans (Silver and Lundgren, 1968; Tabita et al., 1969). Rhodanese is a soluble enzyme. The

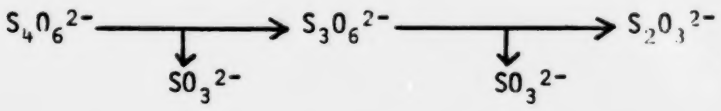
FIGURE 4



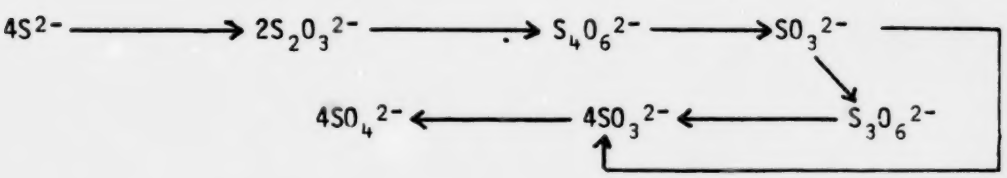
Phylogenetic relationships of the thiobacilli and related purple photosynthetic bacteria. The illustrated network was deduced by the matrix method (10), omitting regions of terminal length variation and ambiguous sequence alignment (see Fig. 1) from homology calculations. 5S rRNA sequences used are given in Fig. 1 (this paper), taken from the literature (7, 9, 33), or are unpublished (*Chromatium*, L. B. Lablin, C. R. Woese, and G. E. Fox; *Pseudomonas diminuta*, *Pseudomonas cepacia*, and *Rhodopseudomonas gelatinosa*, G. E. Fox and K. R. Luehrsen, personal communications). Base-paired regions were assigned one-half the weight of unpaired regions, and alignment gaps were assigned one-half the weight assigned to a nucleotide in the same position. In determining the optimal tree, the mean square difference between the estimated evolutionary distance separating each pair of sequences and the corresponding tree distance was weighted by the statistical uncertainty of the distance estimate (16). An iterative program, following the path of "steepest descent" in the optimization parameter, was used to determine the tree topology which best fit, by the weighted least-mean-square difference criterion, the input homology data. The scale bar represents an evolutionary distance of 0.1 Knuc (average number of nucleotide changes per sequence position). The root of the tree probably lies between groups I and II (dashed segments), as discussed in the text. The inset illustrates the relative position of the purple photosynthetic bacterial within the eubacterial kingdom (32).

(From Lane et al., 1985 with permission)

S-S bond of thiosulfate is split to form S° and SO_3^{2-}. The elemental sulfur is believed to exist as a transient membrane associated sulfur sol or persulfide. This may give rise to the accumulations of sulfur observed microscopically by Gromova et al. (1983). Thiosulfate cyt. c oxidoreductase catalyzes the oxidative condensation of two molecules of thiosulfate to form tetrathionate (Silver, 1978). The metabolism of tetrathionate is then proposed to proceed as follows:



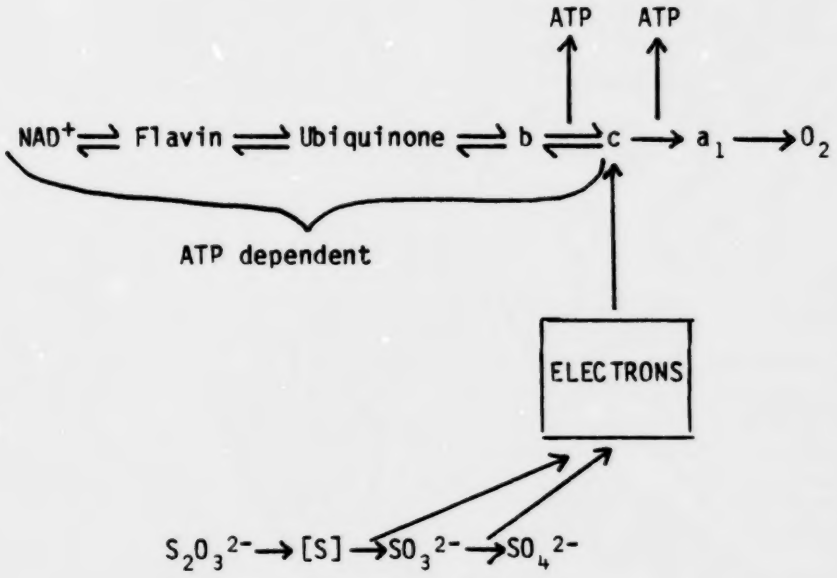
The detection of polythionates in the culture medium lends support to this pathway. It is worth remembering that polythionates are able to form spontaneously from reduced sulfur compounds (Kelly, 1982) as shown below:



Although this polythionate pathway is no longer a credible major pathway for sulfur oxidation, its contribution to sulfur sequestering may be significant.

2. Electron Transport Components

The oxidation of all sulfur compounds in T. ferrooxidans is cytochrome mediated. Electrons enter the chain at the level of cyt. c thus:



The short chain from cyt. c to cyt. a_1 is believed to constitute the usual phosphorylation site in T. ferrooxidans. The longer chain leading to the reduction of NAD^+ is present at much lower concentrations and constitutes the energy dependent reverse electron flow. Ingledew and Cobley (1980) estimated that 20 e^- flow to O_2 for every 1 e^- that goes to NAD^+.

3. Growth

Data derived specifically from the growth of T. ferrooxidans on sulfur compounds is very sparse. The available information indicates that the

growth rate on sulfur compounds is significantly slower than on ferrous iron. Thus Tuovinen and Kelly (1974) estimated the maximum specific growth rate (μ_{max}) to be 0.043 hr^{-1} and 0.05 hr^{-1} on thiosulfate and tetrathionate respectively. This contrasts with a μ_{max} of 0.107 hr^{-1} for growth on $FeSO_4$. Eccleston and Kelly (1978) found a similar μ_{max} on tetrathionate of 0.092 hr^{-1}. With elemental sulfur as substrate, Unz and Lundgren (1961) produced a figure of 0.03-0.07 hr^{-1}; McGoran et al. (1969) could only observe an exceedingly slow rate of approximately 0.004 hr^{-1}.

Calculations of yield however show the opposite trend. Yields (grams [dry wt.] per gram-molecule substrate) on tetrathionate and thiosulfate range from 9.8-12.2 and 5.9-7.5 respectively (Eccleston and Kelly, 1978; Tuovinen and Kelly, 1974). The latter authors, in the same study, could only obtain a yield of 0.35 on $FeSO_4$. Free energy calculations based on redox potentials and the number of electrons transferred during oxidation, indicate significantly more energy available from sulfur compounds than iron. For instance $\Delta G^{\circ\prime}$ (kcal./mole) for $S^0 \rightarrow SO_4^{2-} = -140.6$; $Fe^{2+} \rightarrow Fe^{3+} = -17.0$ (Brock et al., 1984). Beck and Shafia (1964) demonstrated that the efficiency of CO_2 fixation on sulfur compounds was far higher than on iron. The ratio of CO_2 fixation to O_2 uptake was approximately 2 on Fe^{2+} but approximately 20 on S^0. Data on P/O ratios, although fragmentary because they were obtained from a range of different species, support this proposition. P/O values of 0.8 and 0.9 have been calculated with T. neapolitanus and T. novellus oxidizing $S_2O_3^{2-}$; a P/O ratio on 1.4 was obtained from T. novellus oxidizing S^{2-} (Aleem, 1977).

4. pH

Generally the pH range or optimum demonstrated by T. ferrooxidans growing on sulfur compounds is higher than on iron. The pH optimum for tetrathionate growth = pH 2.5 (Eccleston and Kelly, 1978); for S° = pH 5.5 (Cox and Brand, 1984). The pH range for growth on thiosulfate = pH 3.6-4.7 (Tuovinen and Kelly, 1974). Isolated enzymes show an even higher pH optimum for in vitro assays than for in vivo growth; thiosulfate oxidase, pH 5.0 (Silver and Lundgren, 1968), rhodanese, pH 9.0 (Tabita et al., 1969). Rao and Berger (1971) indicated that the constraints on the growth of T. thiooxidans on S° (growth only possible below pH 4.3) were not due to the pH characteristics of the enzymes. Sulfur oxidase was active over the pH range 0.9-7.0.

5. Enzyme Expression and Concentration

The ability of T. ferrooxidans to oxidize Fe^{2+} is considered to be invariate. In contrast, the ability to oxidize sulfur compounds can vary with the state of the culture and the past growth regime. Beck and Brown (1968) found that aged cultures of T. ferrooxidans lost the ability to oxidize S^0 but never lost Fe^{2+} oxidizing capacity. Tuovinen et al. (1976) could not find any activity of the thiosulfate oxidizing enzyme when grown on Fe^{2+}. Likewise in studies on O_2 uptake on sulfide minerals, Duncan et al. (1967) and Silver and Torma (1974) found that the activity was influenced primarily by the substrates the cells had been grown on. Margalith et al. (1966) found that S^0 grown cells had a better capacity for oxidizing S^0 than iron grown cells. When cells were transferred from iron to sulfur they did

not see any lag in oxidation and therefore stated that the sulfur oxidizing enzymes were not inducible. Unz and Lundgren (1961) however, did observe diauxie in the growth of T. ferrooxidans on a mixture of iron and sulfur substrates.

D. CO_2 FIXATION

T. ferrooxidans fixes CO_2 with a fully functional Calvin-Benson cycle (Gale and Beck, 1967; Maciag and Lundgren, 1964). D-Ribulose-1,5 bisphosphate carboxylase (RuBPCase) and phosphoribulokinase (PRK) activity have been localized in polyhedral inclusion bodies (Katayama Fujimura et al., 1984). Such carboxysomes are therefore believed to be the sites of CO_2 fixation. CO_2 limitation in T. neapolitanus is known to increase the amounts of RuBPCase to 17% of cell protein as compared to 4-5% when growth is limited by thiosulfate (Kuenen and Beudeker, 1982). Kelly (1971) reported that PRK was inhibited by AMP.

The Calvin-Benson cycle in T. ferrooxidans operates on its usual requirements of 3 ATP and 2 NADH for every CO_2 molecule fixed (Silver, 1978). The generation of the reduced pyridine nucleotides is hypothesized to occur by ATP dependent reverse electron flow as originally suggested by Aleem et al. (1963). Data are not available on the precise number of ATP's required in T. ferrooxidans to reduce one NAD^+. In T. neapolitanus 2.5 ATP are used per NAD^+ reduced, the figure is 1 ATP per NAD^+ in T. novellus. The oxidizable substrate in both cases was thiosulfate (Aleem, 1977).

Kelly and Jones (1978) demonstrated that the rate of CO_2 fixation was dependent on, and proportional to, the rate of iron oxidation. The CO_2

concentration became growth limiting as the CO_2 supply became exhausted. MacDonald and Clark (1970) tested the effect of CO_2 concentration on iron grown T. ferrooxidans cultures. Between concentrations of 0.03% (normal atmospheric air) to 10% CO_2 (v/v), growth rate was found not to be limited by CO_2.

Eccleston and Kelly (1978) did however observe an increase in apparent cell yield with CO_2 supplementation. Further investigation revealed that this was due to a decrease in the maintenance coefficent in CO_2 rich environments, indicating a more efficient energy coupling with increased concentrations of CO_2.

Calculations suggest that 20 kcal per gram atom carbon is required to fix CO_2, which represents about 20% of the energy yield from Fe^{2+} oxidation (MacDonald and Clark, 1970). Pugh and Umbreit (1966) determined that 10% O_2 v/v was sufficient to support maximum CO_2 fixation. Romanova et al. (1969) stated the maximum CO_2 fixation occured at pH 3.1-3.9. Presumably CO_2 fixation in situ operates sub-optimally.

In addition to the Calvin-Benson cycle, T. ferrooxidans possesses a complete glycolytic sequence, an incomplete citric acid cycle (α-ketoglutarate dehyrogenase is usually absent) and a disputed hexose monophosphate shunt (Ingledew, 1982; Matin, 1978).

E. PSYCHROPHILY

The low ambient temperatures encountered with in place leaching operations have prompted an interest in psychrophilic (optimum temperature < 15°C; maximum temperature < 20°C) or psychrotrophic (growth at 0°C; higher

optimum and maximum temperatures) strains of T. ferrooxidans. All strains of T. ferrooxidans tested to date appear mesophilic with optimum leaching temperatures from 30°C to 40°C and Q_{10}'s = approximately 2.0 (Torma and Gabra, 1977; Lewis and Miller, 1977). Although the bacteria would have to operate at sub-optimum temperatures in Canadian climes, Lazaroff et al., (1982) have indicated that jarosite precipitation is more incomplete at low temperatures and is replaced by a more amorphous form of ferric hydroxysulfate. Hence one impediment would be reduced.

Kovalenko and colleagues are the only workers to have published data specifically on the effect of low temperature on T. ferrooxidans. Kovalenko and Karavaiko (1981) found that decreasing the temperature to 5.5°C inhibited the growth rate far more than the Fe^{2+} oxidation rate. This was accompanied by a considerable increase in K_s for ferrous. The increased energy necessary for growth at low temperature was corroborated by Denisov et al. (1981) who calculated the coefficent of efficiency (CE) of biosynthesis in the range 15-35°C. This coefficent expresses the percentage of energy liberated from the Fe^{2+} and O_2 consumed that is used to construct dry cell biomass. At 15°C the CE decreased to 15% from a high of 35% at 30°C. Denisov et al. demonstrated that lowering the temperature lowers the pH at which ferrous oxidation can occur. T. ferrooxidans was also shown to be more tolerant to both Fe^{3+} and Cu^{2+} at low temperatures (Kovalenko and Karavaiko, 1981; Kovalenko et al., 1982).

The bases of psychrophily have been reviewed by Inniss (1975) and also by Morita (1975). Many areas of cell physiology and morphology are implicated. There are many papers attesting to the increased thermolability

of psychrophilic ribosomes, tRNA species, synthetases and numerous catabolic and biosynthetic enzymes. Membranes of psychrophilic bacteria possess higher concentrations of unsaturated fatty acids and have shorter chain lengths or more methyl branching (Foot et al., 1983; Levin, 1972). Such a variety of systems prompted Morita to state that there are probably many pathways leading to psychrophily and the data is not available to postulate the exact genetic basis.

Whole cell mutagenesis has been successful in elevating the cardinal temperature of some psychrophiles into the mesophile range. There is however only one report of a shift in the opposite direction (Olsen and Metcalf, 1968).

The identification of an inducible desaturase in bacteria which is responsible for the fatty acyl unsaturation is probably one system that could be amenable to cloning. Increased dosage of desaturase might enhance membrane fluidity at low temperatures. The only other identifiable gene product which had a profound influence on psychrophilic growth was a proteinaceous ribosome activating factor (Factor P) isolated from a psychrophilic Pseudomonas sp. by Szer (1970). Factor P allowed cell free poly-U translation at 0°C with the psychrophilic pseudomonad and with mesophilic E. coli extracts. Nothing has been reported on this factor since 1970.

Isolations of bacteria from any permanently cold environment have always produced psychrophiles. Moshnyakova and Karavaiko (1979) found T. ferrooxidans in ores at temperatures from -1.0 to 35-40°C. Harrison (1984) has indicated that several extant strains of T. ferrooxidans can grow at 5°C.

Temperature obviously exerts a very powerful natural selective force. Ecological studies have even indicated that seasonal temperature variations can shift the cardinal temperatures of bacterial populations (Sieburth, 1967).

F. MINERAL LEACHING

1. In Situ Conditions

Razzell (1962) identified five conditions that would allow rapid bacterial leaching of metallic sulfides from ores. These were:

a. a pH of 2.0 to 4.0 at the mineral surface,

b. detectable amounts of Mg^{2+}, NH_4^+, PO_4^{2-},

c. an adequate supply of air,

d. a temperature between 4° and 37°C,

e. a large bacterial population.

Over the years the metallic sulfides that have proven amenable to bacterial leaching have included the sulfides of iron, copper, tin, antimony, gallium, arsenic, cadmium, cobalt, lead, nickel and zinc (Lundgren and Silver, 1980). Iron is invariably associated with these ores which makes it difficult to determine if oxidation of the sulfide occurs directly by the bacterium or indirectly through the ferric ion. Dissolution of copper and uranium from their respective sulfides and oxides has a predominately indirect mechanism. Sakaguchi *et al*. (1976) determined the required Fe^{3+} concentration for maximum chalcocite and covellite oxidation to be 0.004-0.01M and 0.004-0.02M respectively. Conversely stannous ion oxidation is believed to be a direct oxidation (Lewis and Miller, 1977). Torma (1971) demonstrated the

microbiological oxidation of synthetic cobalt, nickel and zinc sulfides in iron-free solutions. In most instances however, direct and indirect oxidation are undoubtedly occurring in concert.

In field conditions a few investigators have noted limitation of growth, and hence metal dissolution, in T. ferrooxidans due to limiting nitrogen and/or phosphorus (Roy and Mishra, 1981a; Lundgren and Silver, 1980). In an extensive and protracted study of its T. ferrooxidans strain, B.C. Research determined that 200 mg/l NH_4^+ and 100 mg/l PO_4^{3-} were necessary for maximum leaching (progress report #24, 1968). McCready et al. (1986) indicated that lower concentrations were sufficient; 30 mg/l MH_4^+ and 10-20 mg/l PO_4^{3-}. Tuovinen et al. (1979) contended that trace amounts of ammonia in the ore would be adequate to satisfy the 0.2mM NH_4^+ requirement of T. ferrooxidans. They did however agree that ammonia supplementation was effective in accelerating the leaching of copper, zinc and nickel. They postulated that this was possibly due to some metal complexation. B.C. Research also reported that increasing the O_2 concentration to 50-52% $^V/_v$ produced optimal leaching on chalcopyrite (progress report #20, 1966).

2. In Situ Populations

The indigenous bacterial population on mineral ores is rarely a pure culture of T. ferrooxidans. Karavaiko et al. (1984) found significant numbers of T. ferrooxidans, L. ferrooxidans and Sulfobacillus thermosulfidooxidans in a copper-zinc-pyrite heap leach at Nikolaev. In many mining situations, because of the exothermic nature of pyrite oxidation, a thermophilic population establishes itself. Thus Sulfolobus type bacteria

are often cited (Chakraborti and Murr, 1979). There is a dynamic succession of bacterial species associated with sulfide ores (Ralph, 1979). The key parameter influencing these population shifts is pH. Norris and Kelly (1978b) established the desirability of using mixed cultures by showing that iron oxidation was greatest in natural mixed populations which had been enriched on pyrite.

G. SUMMARY AND PROSPECTS FOR GENETIC ENGINEERING

The data on T. ferrooxidans indicate that iron oxidation is capable of sustaining a faster growth rate than sulfur oxidation. In order to sustain the high exponential rate of oxidation, the ferrous oxidation must be coupled to growth. The linear rates produced by standing crops of bacteria are far inferior. Attention must therefore be focused on the encouragement of a high intrinsic rate of multiplication in the leaching population.

Information available on CO_2 suggests that only under chronic shortage can CO_2 fixation become growth limiting. Similarly the data on the concentration of O_2 and N/P nutrients indicate that T. ferrooxidans possesses highly efficient scavenging and uptake mechanisms for these key nutrients. There seems little point in trying to improve the microorganism in these areas. The observations from pilot scale studies that increased levels of CO_2, O_2 and N/P improve the leaching rate are believed to be overcoming an engineering problem of maintaining an adequate level of nutrients.

If T. ferrooxidans is usually energy limited, is there any prospect for improving the oxidation pathways per se? Any modifications in the highly structured membrane are presently beyond the scope of present day

biotechnology. This is particularly true when dealing with a vectorial arrangement as seen in the cytochrome chain of T. ferrooxidans. It might however be possible to boost the components in the periplasm. For iron oxidation this would mean rusticyanin and cyt. c. Ingledew (1982) has already determined that rusticyanin/cyt. c comprise 5-10% of cell protein in T. ferrooxidans. Such amounts may be saturating.

There may be more scope with the sulfur enzymes. Groudeva et al. (1981) have reported increased sulfur oxidation in T. ferrooxidans strains by mutagenesis with U.V., ethylenimine and N-methyl-N'-nitro'N-nitosoguanidine. Although the larger biomass that sulfur oxidation can support is irrelevant to a leaching situation, the faster solubilization of sulfide moieties and the removal of any S° film would be advantageous. Again compounds in the periplasm would be the easiest to manipulate. The obvious choice would be sulfide oxidase. This enzyme will have to be purified and characterized before any work can proceed. In T. concretivorus increased levels of polysulfide binding protein might accelerate sulfide oxidation. Unfortunately basic research about this protein in T. ferrooxidans is missing. Generally more elucidation is required on the control of the sulfur enzymes in T. ferrooxidans, especially regarding their expression and activity.

A vital element missing from these speculations is information on the structure and role of the outer envelope in T. ferrooxidans. Although this section has focused on the enzymes and proteins in the periplasm and the cytoplasmic membrane, substrates and products have to be transported through the outer envelope. The role of this structure must be especially important

when the organism is growing on solid mineral sulfides. When information is produced, discoveries about transport pertinent to iron and sulfur oxidation may arise that will change the entire picture.

The literature search into the basis of psychrophily revealed a trait that was multifaceted and undoubtedly coded for by more than one gene. Attempts to induce psychrophilic growth by manipulating a single gene are improbable. It was not even possible to identify a candidate gene in T. ferrooxidans. A cloning approach may be inappropriate for another reason; isolates of T. ferrooxidans have already been reported which are certainly psychrotrophic if not psychrophilic. A course of continued selection at low temperatures would probably be the most successful way of improving this trait.

Finally mention has been made of the tenacity of contaminants to stay in cultures of T. ferrooxidans. This is frustrating for laboratory studies on the physiology of T. ferrooxidans. However the fact that acidophiles show such a propensity to exist in close association is probably a mechanism to ensure the optimal population for their acidic niche. Preliminary work supports this view. Mixed cultures are more avid at leaching minerals and desulfurizing coal (Norris and Kelly, 1978a; Dugan, 1978). T. ferrooxidans is definitely the key member of acidophilic leaching populations. In addition to continuing research on pure cultures of this species, experimentation with mixed cultures may produce rewarding results for the mining industry.

CELL SURFACE AND ADHERENCE

A. INTRODUCTION

The commercial relevance of genetically engineering T. ferrooxidans is in the creation of strains which will more efficiently remove metals from ore bodies. The objective may be to increase the rate of leaching, to expand the range of environments in which leaching will occur, or to increase specificity for a particular metal.

Clearly identifying the objective is critical because each involves a specific aspect of cellular metabolism or function and consequently the traits which have to be engineered will be different.

The metabolic activity which results in solubilization of a metal occurs in four distinct environments each of which presents special problems for the genetic engineer. These environments are: the cell cytoplasm, the cytoplasmic membrane, the cell surface, and the extracellular milieu in which cell free microbial products function. It is the purpose of this section to discuss the role of the surface in the growth and leaching activity of T. ferrooxidans and to indicate where possibilities for genetically engineering the cell might occur.

The cell surface will be defined as that portion of the cell external to the cytoplasmic membrane including the capsule and external structures such as fimbriae, fibrillae, and flagella.

B. COMPOSITION OF GRAM NEGATIVE SURFACES

Immediately external to the cytoplasmic membrane is the periplasmic space. It contains proteins which can be released by procedures which do not release cytoplasmic proteins. Externally the periplasm is bounded by the peptidoglycan, a rigid molecule protecting against lysis and determining cell shape. Bound to the peptidoglycan is the trilaminar outer membrane. This is a complex structure consisting internally of phospholipid and externally of lipopolysaccharide (LPS). Contained within this amphiphillic matrix are a variety of proteins. In many organisms the surface is penetrated by hair-like structure known as fimbriae, fibrillae or pili. In their natural environment most organisms produce a carbohydrate capsule which coats the outermost layers of the cell. Motile organisms possess flagella which extend from the cytoplasmic membrane to the exterior environments.

C. COMPOSITION OF T. FERROOXIDANS

The limited number of studies done on the composition of the Thiobacillus suggest that they conform to the pattern observed for most Gram(-) organisms. A trilaminar outer membrane and a thin peptidoglycan layer was observed by Wang et al. (1968; 1970). Ultrastructural studies of other Thiobacilli indicate a similar cell surface structure (Shively et al., 1970; Holt et al., 1974). Pili have been reported in Sulfolobus (Weiss, 1973) and T. kabobis (Reynolds et al., 1980). Chemically the outer membrane of T. ferrooxidans has been shown to consist of protein, carbohydrate and lipid. The LPS contained heptose, 2-keto 3-deoxyoctanoic acid, glucose, galactose and mannose (Wang et al., 1970). Very low levels of phosphate

were detected. The toxic and immunological properties of T. ferrooxidans LPS have been studied (Vestal et al., 1973). The peptidoglycan has been characterized by Wang et al. (1970). It is probable that these studies on the chemical characterization were conducted with mixed cultures of acidophillic organisms. Care should be taken in interpretation of the data when referring to growth on different substrates. The relationship between the structure of the Thiobacillus cell surface and its role in the solubilization of sulfur and iron from crystalline material is discussed by Lundgren and Tano (1978). Virtually nothing is known about the protein composition of the outer membrane of the organism. Recently it was reported that growth on iron induces formation of some proteins, including rusticyanin, a protein believed to be located in the periplasm (Mjoli and Kulpa, 1986).

In summary the microscopic and analytical data available at this time indicate that the surface layers of T. ferrooxidans are similar to other Gram(-) organisms. Identification of the unique features which enable the organism to survive in extremely acidic environments awaits a more detailed analysis of the molecular structure and organization.

D. FUNCTION

The outer layers of Gram negative organisms serve a variety of functions.

1. Adherence

Adherence is now recognized as an essential ecological determinant for organisms which are subjected to the cleansing action of flowing water or the

detergent action of particulate matter. Organisms may attach via hydrophobic, ionic, electrostatic and lectin-like interactions. For a review of the physiochemical nature of forces holding bacteria to substrata the recent article by Rutter and Vincent (1984) is recommended. The role of polymeric substances in binding is discussed by Robb (1984). Of specific interest is the review of the nature of the interactions involved in binding to inanimate surfaces (Kjelleberg, 1984). In most instances it is thought that irreversible attachment is the result of a combination of different forces. Binding is often a highly specific reaction involving unique cell surface components (adhesins) which recognize a distinct receptor. Once this specific association has been made the attachment may be strengthened by less specific interactions. In some cases this will involve synthesis of extracellular polymers at the attachment site. The glycocalyx (Costerton and Irvin, 1981; Bryant et al., 1984) is a good example of an extracellular cementing substance which has been found on many adherent organisms.

Cell surface constituents implicated in adherence to living and non-living surfaces include proteins, glycoproteins, carbohydrates and amphiphillic substances such as LPS. These compounds may be organized into (or associated with) specific structures such as fimbriae or they may exist as discrete entities within the matrix of the cell surface. Pili, fimbriae or fibrils are frequently involved in specific binding reactions. Often a cell will contain a number of chemically distinct fimbriae each capable of recognizing a unique receptor molecule. Phenotypic and in some cases genotypic analysis indicate that expression of these fimbrial adhesins is controlled by the growth environment (Silverman et al., 1984).

The release of minerals and oxidation of sulfur and iron by T. ferrooxidans has been related to the capacity of the organism to make intimate contact with the mineral substrate (Bennet and Tributsch, 1978; Duncan et al., 1964; Torma et al., 1976). There are however examples where unattached organisms effectively oxidize iron (Harrison, 1984; Wakao et al., 1984). Attachment to the substrate serves two purposes: (1) the organism is brought into close association with the substrate and (2) the organism is resistant to removal by hydrodynamic forces.

Studies concerned with the effects of wetting agents on the rate of iron and sulfur oxidation bear indirectly on the question of the importance of attachment as it is probable that these substances influence the interaction of the bacterium and the mineral. Duncan found that compounds such as Triton X-100 enhanced leaching of chalcopyrite and suggested that this was due to a closer contact of the cell with the mineral (Duncan et al., 1964). This observation was refuted by Torma et al. (1976) who observed decreased chalcopyrite oxidation and attributed this to inhibition of binding of bacteria. While the two groups obtained contradictory information, both stress the importance of intimate contact with the mineral. Wakao et al. (1984) reported that Tween 20 enhanced pyrite oxidation by disrupting the binding of the cell to pyrite. Cells in suspension were more proficient at oxidizing iron than tenaciously bound cells. They also propose that enhanced pyrite oxidation caused by the addition of T. thiooxidans to T. ferrooxidans cultures may be due to the wetting agents (Schaeffer and Umbreit, 1963; Shively and Benson, 1967) secreted by the firmly bound T. thiooxidans (Baldensperger et al., 1974; Takakuwa et al., 1979. The wetting agent would

selectively release T. ferrooxidans which would be more proficient at oxidizing iron.

T. ferrooxidans binds to a variety of substrata including glass, coal, sulfide containing minerals, sulfur, quartz and fluorapatite (Wakao et al., 1984; Bennet and Tributsch, 1978; DiSpirito et al., 1983; Myerson and Kline, 1983; Lundgren and Tano, 1978; McGoran et al., 1969; Gormely and Duncan, 1974; Keller and Murr, 1982; McCready and LeGallais, 1984; DiSpirito et al., 1981; Murr and Berry, 1976). Much of this work has been descriptive in nature with analysis made by direct scanning electron microscopic observation of binding. This technique is not particularly useful for studying the kinetics of binding because of the difficulty in accurately measuring the number of bound cells. Quantitative data have been obtained by measuring the numbers of ^{32}P labeled cells bound (McCready and LeGallais, 1984) or by measuring the biomass associated with the particulate matter (DiSpirito et al., 1983; Gormely and Duncan, 1974). In the latter, the amount of bound bacterial protein served as the indicator of cell mass. Wakao et al. (1984) have shown that almost all cells were rapidly bound to finely divided pyrite or the glass walls of the assay vessel. Gormely and Duncan (1975) showed that more than half of the cells added bound to zinc sulfide. DiSpirito et al. (1983) measuring the loss of protein from the liquid phase found that binding was rapid. Twenty-five percent of cells bound in the first 5 minutes with a steady state achieved within 1 hour. Myerson and Kline (1983) determined the effect of cell numbers on binding to coal and glass. They found that at low cell concentrations virtually all bacteria were bound to the solid within 30 minutes; binding was irreversible.

Direct observation has shown that cells bind to a limited area on the pyritic material (Wakao et al., 1984) indicating that the pyrite surface is heterogenous with respect to its ability to bind bacteria. This suggests that specific mineral configurations or mineral compositions favour attachment by providing a receptor which the cell recognizes. Lundgren and Tano (1978) have suggested that cells attach to the mineral sulfide. McCready and LeGallais (1984) found that ^{32}P labeled T. ferrooxidans bound to the mineral ash and pyritic components of coal but did not associate with hydrophobic organic constituents.

Long term exposure of sulfide minerals to T. ferrooxidans leads to the formation of bacterially etched pits (Bennet and Tributsch, 1978; Keller and Murr, 1982; Baldensperger et al., 1974; Tributsch, 1976). Bennett and Tributsch (1978) hypothesize that the organization of bacteria on a pyrite surface is dependent on crystal order. This view is disputed by Keller and Murr (1982) who did not find that colonization by bacteria was restricted to a particular crystal organization.

The molecular basis of attachment of these organisms is poorly understood. Takakuwa et al. (1979) reported that T. thiooxidans bound to sulfur particles via bacterial sulfhydryl groups. The reaction was pH and energy dependent and could be blocked by sulfhydryl-binding reagents. These results have been questioned (Bryant et al., 1984). It would appear unlikely that sulfhydryl bonding is involved. Byrant et al. (1984) have shown that T. albertis binds via a glycocalyx to sulfur discs. DeSpirito et al. (1983) reported that the removal of LPS decreased binding to pyrite, flower of sulphur and flouroapatite. It should be noted that the LPS extraction

procedure would have removed many other cell surface constituents. Wakao et al. (1984) found that dead cells bound as rapidly as living cells to pyritic minerals. Exposure of cells to ultra-violet irradiation did not affect the initial rates of adsorption but did decrease the total number of bound cells. This review points out the paucity of studies designed to characterize the molecular nature of the T. ferrooxidans mineral binding reaction. Those studies which have been done provide very little information which could be used to identify the bacterial structures which mediate binding.

Another aspect of T. ferrooxidans adherence which has received very little attention is its ability to bind to other organisms or into an organic matrix. The observation that streamers in mine water draining from a pyrite mine were populated by T. ferrooxidans embedded in a gelatinous matrix suggests that at least some strains possess the capacity to exist in large aggregates held together by organic polymers (Wakao et al., 1985).

The literature on the adherence of T. ferrooxidans clearly shows that the organism binds avidly to a variety of mineral surfaces. There is some indication that binding to minerals is a specific process restricted to certain areas of the mineral surface. There is conflicting information on the importance of binding in the mobilization of iron and sulfur. The question remains as to whether binding is an essential requirement in the release of oxidizable substrates? Notwithstanding the lack of consensus on the necessity of binding, it is a reasonable assumption that in natural and heap leaching processes bacteria are in intimate contact with the surface they are degrading. Based on our knowledge of other adherence processes this

is probably a specific reaction dependent on unique constituents (adhesins) on the surface of the cell. The manipulation or elimination of these adhesins will help to resolve the question of the role of adhesion in mineral leaching and will create the opportunity to determine if the organism has the capacity to recognize different receptors on the mineral surface.

Regardless of whether adherence is required for oxidation of the minerals it may be essential to retain the organisms within the ecosystem and/or to create the micro-environment required to establish a leaching reaction. The same requirements might not be needed in a fermentor leaching system where washout would not be a problem and where it is possible to non-biologically create an environment appropriate for oxidation and leaching.

2. Metabolism

In addition to adherence the outer layers serve to facilitate transport of some solutes through the outer membrane (porins), and restrict entry of other molecules. Enzymes involved in synthesis and in nutrient acquisition are located in the periplasm and outer membrane. Other proteins serve to bind nutrients during transit to the cytoplasmic membrane. Some surface components may detoxify by binding toxic metals.

While little is known about the molecular and functional properties of the outer layer from T. ferrooxidans it is reasonable to assume that it plays a unique role in the growth of the organism. A list of possible properties and functions would include:

a. Mobilize and transport reduced forms of sulfur, possibly in an insoluble form.

b. Transport large amounts of Fe++ to the cytoplasmic membrane.

c. Transport large amounts of Fe+++ to the cell exterior. (Fe+++ is a highly insoluble compound and would rapidly clog the cell surface.)

d. Transport large quantities of sulfate away from the cell.

e. Must be stable at low pH. The inference is that any protein engineered into the wall would have to have acid resistant properties, or exist in an environment protected from the acid.

E. SUMMARY AND PROSPECTS FOR GENETIC ENGINEERING

Given this background information it is appropriate to consider how genetic engineering might be used to modify cell surfaces. Two objectives to be discussed are: (a) increase the rate of leaching, (b) increase selectivity for a specific metal.

If the rate at which a metal is solubilized is controlled by an event which occurs externally to the cytoplasmic membrane it may be possible to increase the rate of leaching by increasing the speed of the rate limiting reaction. Examples of potentially rate limiting reactions include:

i. Mobilization of the insoluble mineral at the organism-mineral interface

ii. Transport of Fe++ and/or reduced sulfur

iii. Oxidations of reduced sulfur

iv. Removal of Fe+++ from the periplasmic space

If adherence is a specific process associated with unique adhesins it should be possible to manipulate the adhesins to create strains with enhanced abilities to bind to specific mineral surfaces. In this way organisms could be engineered for specific types of mineral deposits.

The conclusion that can be reached from this brief review of the surface related activities of T. ferrooxidans is that it would not be wise to embark on a program of genetic engineering until more is known about the outer membrane and adherence properties of the organism.

Suggestions for study:

i. Identify adhesins and determine their role in binding to different minerals. Identify a role for adhesins in leaching.

ii. Identify components involved in the transport of iron and sulphur.

iii. Study the organization and location of the transport and oxidation systems.

HEAVY METAL RESISTANCE

A. INTRODUCTION

This section will consider three aspects of heavy metal resistance: levels of heavy metals one might expect; the toxicity of heavy metals to T. ferrooxidans or near relatives, and relevant data on the resistance of other organisms to the heavy metals with particular reference to the mechanisms of resistance where possible. There is an excellent review on metal resistance in bacteria by Trevors et al. (1985). The information on mechanisms will focus on whether the observed resistance is significant relative to the levels expected in the T. ferrooxidans environment and whether the mechanism of resistance is energy dependent. If the resistance is energy dependent, then extreme levels of resistance could have a significant and negative effect on the growth rate of the bacteria. Such mechanisms may not be as useful as ones which reduce membrane permeability.

Tuovinen et al. (1971) point out that the effect of some heavy metals depends on the energy source being oxidized. When oxidizing iron T. ferrooxidans was able to tolerate very high levels of metal ions, whereas when oxidizing thiosulfate the tolerances were greatly reduced, sometimes up to 2000 fold. Finally, when oxidizing sulfur, the tolerance to metal ions was intermediate but closer to the levels found when iron was being oxidized for growth. For example, when oxidizing Fe^{+2} the strain of T. ferrooxidans used could tolerate in excess of 10 g/L Zn, Ni, or Cu. When grown on thiosulfate the inhibitory levels were: Zn, 600 mg/L; Ni, 150 mg/L and Cu, 5 mg/L. When grown on sulfur flower, the inhibitory levels for these three

metal ions were above 5 g/L. Silver and Torma (1974) also have reported data which may bear on the effects of the substrate on metal resistance. Thiobacillus cultures were grown on different substrates and then tested for oxidation rates on different metal sulfides over the course of several hours. Cells grown on $FeSO_4$ or on $CuFeSO_4$ were very similar and could oxidize pyrite and chalcopyrite readily while they were inefficient at oxidizing NiS, SbS_3, CuS, CoS, Cu_2S, PbS or galena. Cultures grown on PbS showed very similar patterns of oxidation except they were inefficient at pyrite oxidation and much more efficient at PbS and galena oxidation, the latter being oxidized at the same rate as chalcopyrite. A paper by Imai et al. (1975) has a great deal of data, however, the culture used in the studies is almost certainly not a single T. ferrooxidans strain.

B. DATA ON THE EFFECTS OF HEAVY METALS ON T. FERROOXIDANS

1. Arsenic Resistance

The level of arsenic in leaching conditions is around 12 g/L at a maximum (Gormely, in-house data). Furthermore, there would be significantly less AsIII than AsV at low pH. Tuovinen et al. (1971) indicated that a level of 1.5 mM of a mixture of arsenate and arsenite would inhibit iron oxidation so that the level of these ions could be significant.

Arsenate resistance in Gram negative bacteria is well known. Resistance to 10 g/L for sodium arsenite and 4 g/L for sodium arsenate are established (Smith, 1978; Silver and Keach, 1982; Hendrick et al., 1984). These resistances are plasmid mediated. The resistance to arsenate is distinct from that for arsenite but the latter also confers resistance to antimony

III. All three resistances are inducible by any one of the three ions. The mechanism for arsenate resistance involves a membrane protein and the expense of high energy phosphate. Such a mechanism might create a metabolic drain to effect arsenate resistance. The mechanisms of arsenite and antimony resistances are not understood.

2. Chromium and Boron

Chromium and boron do not form sulfides although Cr is associated with iron in some minerals. T. ferrooxidans is resistant to 15 mM $Cr_2(SO_4)_3$ and is partially inhibited by higher levels. 10 mM $K_2Cr_2O_4$ causes 50% inhibition of growth rate and 100 mM was strongly inhibitory although the latter may be due to chemical oxidation of the substrate Fe^{+2} (Wong et al., 1982). The sensitivity to boron has not been tested. These two metals are grouped because the resistance to these two ions has been shown to be plasmid linked in Pseudomonas (Summers and Jacoby, 1978; Bopp et al., 1983). The level of resistance to chromate is quite high reaching 1 g/L while the borate resistance is in the millimolar range. Neither mechanism has been examined.

3. Cadmium

The level of Cd which might be expected during leaching is in the range of 200 to 300 mg/L. Imai et al. (1975) indicated that iron oxidation and growth of their strain of T. ferrooxidans was normal to 10mM. Silver and Dinardo (1981) reported that growth on thiosulfate of a stock T. thiooxidans strain was inhibited by 0.2 mM (40 mg/L). A strain from a leach operation was about 10 times more resistant when oxidizing thiosulfate and 3 times more

resistant when oxidizing tetrathionate than was the standard strain under the same growth conditions.

Resistance to Cd has been linked to plasmids in several organisms including Staphylococcus (El Solh and Erlich, 1982) and Pseudomonas (Marques et al., 1979). The resistance genes are often found associated with mercury resistance and penicillinase resistance (Marques et al., 1979; Nakahara et al., 1977a, b). The resistance is due to an efflux mechanism in Pseudomonas (Tynecka et al., 1981) and is energy dependent. Aiking et al. (1985) reported that for cultures of Klebsiellia, detoxification of Cd may be related to the formation of insoluble salts with phosphorus or sulfur.

4. Copper

Data from leach operations (Murr, 1980) suggest that the levels of Cu in dump leaches would be about 1-2 g/L (0.01-0.02 M) whereas in a fermentation operation Cu might approach 50 g/L (0.9 M) in order to produce a suitable feed for electrowinning (McElroy and Brunesteyn, 1978).

Toxicity of copper under laboratory conditions is evident at the lower of the levels listed above. Imai et al. (1975) reported inhibition of their cultures at 10 mM $CuSO_4$, while Tuovinen et al. (1971) reported no inhibition at 0.5 mM during growth on iron. These levels seem rather low considering the evident growth of T. ferrooxidans in leaching conditions. Tuovinen et al. (1971) reported that during growth on sulfur, copper did not inhibit growth up to 70 mM. The maximum level of copper tolerated was not reported by Tuovinen et al. (1971): the data was given as "greater than".

Thiosulfate grown cells were not tested by Tuovinen et al. (1971) because of precipitation of the copper.

There have been reports of copper resistance linked to plasmids but none of the copper resistances are above the basal level for T. ferrooxidans For example the plasmid in E. coli confers resistance to 20 mM (Tetaz and Luke, 1983). Bitton and Freihoffer (1978) suggested that the cell capsule might play a role in Cu resistance.

5. Mercury Resistance

There is some question as to whether mercury would be a significant inhibitory factor in leaching operations because of the high levels of sulfur compounds which would complex free Hg. Nevertheless there are data on mercury resistance in T. ferrooxidans. Imai et al. (1975) indicated that 10^{-4} M would completely inhibit iron oxidation and growth. Norris and Kelly (1978) originally suggested that T. ferrooxidans was very sensitive to Hg and described Hg resistant strains which grow in the presence of 5 μM Hg. The resistant strains carry mercuric reductase (Olson et al., 1982) which is very similar to the one found on plasmids in Gram negative bacteria (Booth and Williams, 1984). Whether the mercury resistance in T. ferrooxidans is plasmid associated is not known.

6. Molybdenum

The levels expected for Mo are in the range of 0.11 g/L at a maximum but typical levels would be 0.01 g/L (0.2 mM) (Melis et al., 1983).

Tuovinen et al. (1971) reported inhibition at concentrations less than 0.02 mM and Jack et al. (1983) reported inhibition of T. thiooxidans (ATCC 8085) at 0.01 mM. Kelly (1968) had previously reported that Mo and most other group IV metals inhibit thiosulfate and tetrathionate oxidation in T. thiooxidans.

The occurrence of Mo resistant organisms is known, but the mechanisms are not known. A Pseudomonas culture resistant to approximately 2 mM has been reported (Marques et al., 1979).

7. Silver

It is possible that like mercury, silver would not be a problem in leaching because of the sulfide concentrations and the formation of jarosites with iron.

There have been several studies of silver toxicity on Thiobacilli. Imai et al. (1975) showed that 10^{-3} M gave complete inhibition of growth but 10^{-5} M resulted in a two day delay in growth. This may signal a shift in the bacterial population. Tuovinen et al. (1971) indicated inhibition of oxidation at approximately 10^{-4} M. Silver and Dinardo (1981) found that inhibition of growth of T. thiooxidans on thiosulfate occurred in the range of 10^{-5} M. Bacteria which accumulate silver granules (Ag_2S) have been reported (Pooley, 1981), but it is not clear whether this relates to resistance mechanisms.

Silver resistance up to 5×10^{-4} M has been reported (Sugio et al., 1980) for a mixed T. ferrooxidans culture. Pseudomonas which grow in the

range of 10^{-1} to 10^{-2} M have been described (Belly and Kydd, 1982) and in one case plasmid linked genes have been implicated (Haefeli et al., 1984).

8. Selenium and Tellurium

The expected levels of Se and Te are approximately 0 to 20 ppm (in the range of 10^{-4} M).

Tuovinen et al. (1971) indicated that the inhibitory levels of SeO_2 would be approximately 9 mM and for $NaTeO_3$ approximately 0.5 mM. Thus the natural resistance levels would seem to be adequate.

Bacterial resistance to Se and Te is rather common (Trevors et al., 1985) and there is an example of plasmid linked Te resistance in Pseudomonas (Summers and Jacoby, 1977) to 10^{-3} M.

9. Uranium

Typical levels for U would be in the 0.5 to 1 gm/L (Bruynesteyn and Vizsolyi, 1981) level with 7 g/L (Weir and Masters, 1980) being very high.

Tuovinen et al. (1971) reported values for inhibition of iron oxidation by T. ferrooxidans are approximately 0.7 g/L which is in the typical level range.

There is the report of a soil Pseudomonas species resistant to 1.6 g/L, however, the genetics and mechanisms of resistance are uncharacterized (Marques et al., 1979).

10. Zinc

Strains of T. ferrooxidans (or cultures containing T. ferrooxidans) would appear to tolerate in excess of 10 g/L Zn under laboratory conditions (Tuovinen et al., 1971). Concentrations of Zn in stirred tank leaching experiments have been reported to reach 119 g/L (Torma et al., 1972).

The levels of resistance of T. ferrooxidans to Zn and to Ni, (Tuovinen et al., 1971), are far above those for most organisms. The natural resistance of T. ferrooxidans however is expressed when oxidizing iron but not thiosulfate where the levels for inhibition drop to 600 mg/L for Zn (10^{-3} M). Low tolerance to metals was noted by Imai et al. (1975) during growth on sulfur. Tuovinen et al. (1971) found that T. ferrooxidans could tolerate over 5 g/L Zn when growing on sulfur. In contrast, Silver and Dinardo (1981) reported that T. thiooxidans was inhibited by Zn in the range of 100 mg/L.

The cause of limitation of Zn resistance is not known: the target of Zn inhibition has not been identified and no examples have been reported where resistance is linked to plasmids. At the same time, it is not clear why T. ferrooxidans is so resistant to Zn during iron oxidation.

C. SUMMARY AND PROSPECTS FOR GENETIC ENGINEERING

From the present data there is a group of metal resistances which are worthy of further consideration. Resistance to silver, molybdenum, mercury, selenium, tellurium and arsenate/arsenite all can be carried on plasmids in Gram negative bacteria to levels which approach the maximum amount expected in leaching operations. These plasmids represent a genetic pool which may be

of significant use in engineering Thiobacillus. A key study which needs to be done is the extent to which these ions represent a problem in leaching operations. This information may be available from chemical analyses of leaching liquors and ore. The additional information which will be needed is basic genetic engineering methodology to allow gene expression in Thiobacillus. As indicated in the Genetics section, this methodology faces some serious tests.

It is worth mentioning that resistance to arsenate might be a good genetic marker to use with Thiobacillus since the genes reside on a plasmid already in Pseudomonas and the genetic test can be established without creating primary mutants in Thiobacillus. In addition, cloning of the mercuric reductase from Thiobacillus would be a good entry into gene structure as a considerable amount of enzymology has been done on the enzyme. Mercuric reductase may also serve as a useful genetic marker for Thiobacillus.

Under the present state of knowledge, the potential for developing Thiobacilli more resistant to cadmium, chromate and/or boron is unknown: more information is needed, especially concerning the inhibitory levels of these ions in leaching operations.

Two of the ions, copper and zinc are clearly important, yet there are gaps in the understanding of the mechanisms of resistance. A study of the copper induced inhibition of iron and sulfur oxidation and overall growth is required. The study should use standard and mining site strains, but should concentrate on a standard strain. At present the data on copper inhibition is self contradictory. Information on the mechanisms of Zn resistance, for

example, energy dependence, is required to assess whether the cell can be made to tolerate more Zn.

GENETICS

A. INTRODUCTION

There are two general areas which will be considered in reviewing the genetics of T. ferrooxidans: the state of chromosomal genetics of T. ferrooxidans and related organisms and the current knowledge of extrachromosomal replicons either in T. ferrooxidans itself, or elements derived from T. ferrooxidans and their characteristics in other organisms (predominantly E. coli). In considering the transfer of T. ferrooxidans genes to other organisms, the signals used to maintain and express genes are important. In general there is very little data on the genes and their controls in T. ferrooxidans so that there are many more questions than answers.

B. THE GENOME OF T. FERROOXIDANS AND CHROMOSOMAL GENETICS

An important problem in assessing the literature of T. ferrooxidans is the question of whether the studies of T. ferrooxidans have used pure strains. This problem is discussed in Harrison (1984). Many authors have not paid enough attention to contamination by commensal acidophilic heterotrophs so that one cannot be certain of the conclusions. In the papers reviewed below it is assumed that the cultures used were in fact pure.

The structure of the genome of T. ferrooxidans has been given rather little attention except for measurements of G+C content and the few taxomony studies described below. The G+C content is within the range of most

mesophiles so that one does not expect unusual features from the DNA (Harrison, 1984). Very recent work from D. Holmes (personal communication) has begun to elaborate the features of the T. ferrooxidans genome more fully. The size of the genome is 70% that of E. coli as analyzed by DNA reassociation studies. There appear to be several families of repeated DNA elements. These elements constitute approximately 6% of the genome. The complexity of these elements is uncertain at this time; however, two of the repeated elements have been isolated by cloning (see below) and if they are representative of all of the elements then there may be as many as eight families of repeated elements each present in 20-30 copies in the T. ferrooxidans genome. Different isolates of T. ferrooxidans have different elements. Finally, Holmes has examined the chromosomal locations of one of the repeated elements by hybridization of the cloned element to restriction endonuclease digests of chromosomal DNA from cells maintained under different culture conditions. The hybridization results suggest that the position of the repeated elements may not be strictly maintained. The elements would thus constitute a set of mobile genetic elements. These elements could have significant implications for the stability of the entire genome of T. ferrooxidans.

The repeated elements found by Holmes and co-workers have been analyzed by restriction digestion patterns. Two different elements have been subjected to further study. The elements appear to be about 1 kb in length and vary in frequency of occurrence and in position around the genome. One of the elements has been found on a 45 kb plasmid which is native to this

strain of T. ferrooxidans. DNA sequencing of the repeated elements is under way. The elements were originally isolated during random cloning of T. ferrooxidans fragments in an attempt to define DNA fragments which could be used as molecular probes for identification of T. ferrooxidans strains. Such probes have been isolated and provide an important tool for the testing of strain identity and purity.

A significant limitation in the analysis of the genetics of T. ferrooxidans is the lack of mutants in basic biochemical functions. There have been some reports of mutants (Groudeva et al., 1981; Yankofsky et al., 1983) however the genetic and biochemical defects have not been well described. The problems associated with lack of growth on solid media, which is essential for mutant isolation, need addressing in a systematic manner (Holmes et al., 1983). Holmes (personal communication) has suggested that T. ferrooxidans can be grown on solid media with purified agarose and using thiosulfate as the energy source. There are reports in the literature (see Touvinen et al., 1971; Tuttle and Dugan, 1973; Wakao et al., 1983) that T. ferrooxidans is inhibited by organic compounds, however it is not clear that T. ferrooxidans cannot be grown in the presence of a limiting amount of a required growth factor as might be the case for an amino acid auxotroph with a limiting amount of the required amino acid. There are significant features of T. ferrooxidans which might be used as genetic markers on the chromosome, for example the resistance to heavy metals (see above) or the ability to fix atmospheric nitrogen (MacIntosh, 1978; Barros et al., 1985a). The ability to genetically manipulate T. ferrooxidans would be greatly aided by an

expanded background of knowledge of the basic genetics and biochemistry of the organism.

The taxonomy of T. ferrooxidans has received some attention recently. T. ferrooxidans (ATCC strain 19859) is not closely related to any other Thiobacillus except T. thiooxidans (Lane et al., 1985). The next most closely related strain was found to be T. thioparus which is not acidophilic. The data of Lane et al. (1985) indicate that there are two groups of acidophillic Thiobacilli. T. ferrooxidans and T. thiooxidans (and perhaps T. concretovorus) form one group while T. acidophilis is the only representative of the other group. All of the Thiobacilli which are non-acidophilic and/or are chemo-organotrophic ("facultative") are no more closely related to T. ferrooxidans than is the bacterium Rhodopseudomonas gelatinosa. Harrison (reviewed in 1984) and Katayama-Fujimura et al. (1982) used DNA-DNA hybridization to examine relationships among Thiobacilli. Katayama-Fujimura et al. (1982) grouped T. ferrooxidans ATCC 19859 with T. thiooxidans. However another strain from a "natural isolate" (THI 135) had no homology to other Thiobacilli tested (T. thiooxidans was not examined). These results tend to support those of Lane et al. (1985) that T. ferrooxidans has become significantly separated from other bacterial species including other sulfur oxidizing strains.

Yates and Holmes (1985, 1986) have shown that it is possible to identify T. ferrooxidans using molecular probes since strains of T. ferrooxidans have high homology, but are relatively unrelated even to other Thiobacilli. In addition, it is possible to differentiate between various ATCC strains of T. ferrooxidans using restriction endonuclease fragment polymporphisms.

C. PLASMIDS IN T. FERROOXIDANS

There are a number of well documented reports of plasmids in T. ferrooxidans. Martin et al., (1981) found that 11 of 15 strains of T. ferrooxidans from a variety of sources including several ATCC strains contained plasmids. These plasmids ranged in molecular weight from 15 mD to over 50 mD. Some strains may have multiple plasmids. Holmes et al., (1984a) showed that there are three plasmids in T. ferrooxidans ATCC 33020 (6.7, 16, >23 kb). The three plasmids from ATCC 33020 have very little homology with each other and so probably do not contain a common origin of replication (Holmes et al., 1984a). The 6.7 kb plasmid was cloned into the E. coli vector pBR322 and could be maintained in E. coli. While no obvious protein product could be detected, promoter activity from the DNA originating from the T. ferrooxidans plasmid was detected (Holmes, personal communication).

Rawlings et al. (1984) examined isolates of T. ferrooxidans from a mining operation (strain FC) and found plasmids with molecular sizes 4.5, 12.4, 27.6 kb which are remarkably similar to those found by Holmes et al. (1984a). Rawling et al. (1984) reported that the 12.4 kb plasmid could be cloned into pBR322 and the pBR322 origin of replication could then be deleted without loss of the plasmid, indicating the T. ferrooxidans plasmid contained an origin of replication which functioned in E. coli. If this was a general finding, it would be of considerable significance as it would imply that E. coli replication signals and T. ferrooxidans replication signals are compatible. The report did not give data on the stability of the replicon based on the T. ferrooxidans vector origin. In a continuation of

the studies, Rawlings and Woods (1985) created chimeric plasmids with T. ferrooxidans plasmids and pBR325. Although pBR325 itself is nonmobilizable, the chimeras could be mobilized (that is transferred to other E. coli) by IncP, but not IncI or IncW incompatibility group plasmids. While these data do not directly indicate that there is conjugation in the Thiobacilli, the proteins directing DNA transfer from some Gram negative plasmids can recognize T. ferrooxidans DNA sequences.

D. RECOGNITION OF T. FERROOXIDANS GENETIC SIGNALS IN E. COLI

The fact that the T. ferrooxidans plasmid studied by Rawlings et al. (1984) could supply an origin of replication to an E. coli plasmid provides evidence that T. ferrooxidans sequences may bear some resemblance to those in E. coli. However, not all signals can be expected to be recognized. The plasmids isolated by Holmes et al. (1984a) were not able to supply replication origins. More recently, Barros et al. (1985a) have found that a chromosomal DNA fragment from their T. ferrooxidans strain FC could be cloned into a pBR322 plasmid and used to complement a mutant in E. coli. This suggests that transcription and translation signals are being recognized. If these reports turned out to be general, a considerable amount of the technology developed in E. coli might be transferable to T. ferrooxidans (assuming the difficulties described below for DNA transfer were overcome). This latter point serves to emphasize the significance of developing basic genetics in T. ferrooxidans to make the organism suitable for genetic engineering.

E. DNA TRANSFER IN THIOBACILLUS

For a variety of bacteria, although by no means all bacteria, it is possible to isolate genes from the organism, manipulate them in E. coli and then transfer them back to the original host. In this type of a scheme, as well as when the original host is used for genetic manipulation, a means of transferring DNA into recipient bacteria is essential. There are conflicting reports on the introduction of DNA into Thiobacillus recipients. Barros et al. (1985b) studied transformation of two T. ferrooxidans strains: FD1 which grew mixotrophically with glucose (and thus is suspect as to its classification) and a strain from a mine leach, TF35. They reported that the standard calcium chloride treatment for plasmid transformation of these strains was unsuccessful. Spheroplasting was tried with variable results. Strain FD1 could be used to make spheroplasts and these would subsequently regenerate, however TF35 gave only 40% spheroplasts and these failed to regenerate. Protoplast fusion experiments were not reported. Holmes has also tried the "standard" methods for transformation without success (personal communication). A significant factor in these studies is the lack of a selectable marker, that is a gene which is known to be expressed in Thiobacillus to give a measurable characteristic. There is a report of transformation in Thiobacillus. Yankofsky et al. (1983) reported transformation of Thiobacillus strain Y to naladixic acid resistance. However, strain Y is neither characterized nor is it acidophilic, although it was reported to be a chemolithotroph. The transfer of DNA into T. ferrooxidans could be the limiting technology for genetic engineering in Thiobacillus, but it is not clear whether the problems result from the

acidophilic nature of the organism, the chemolithotrophic character, or simply the lack of a good genetic marker. Rawlings et al. (1984) suggested the arsenate resistance might be a useful marker for transformation to Thiobacillus. Holmes has created a plasmid containing the smallest plasmid from ATCC strain 33020 and the arsenate resistance segment from R46 (from a Pseudomonas plasmid) but he was unable to transfer this into T. ferrooxidans (Holmes et al., 1984b). Holmes (personal communication) suggested that T. ferrooxidans is sensitive to a number of antibiotics which might be used to select resistant derivatives which could then be used as a source of genetic markers in transfer experiments.

F. WORK WITH NON-ACIDOPHILIC THIOBACILLI

As a preface, it is advisable to remember that DNA homology studies indicate that T. ferrooxidans has no more than 3% homology with the other non-acidophilic strains of Thiobacillus and that the relationship to other acidophiles, with the exception of T. thiooxidans, may not be much greater. However some other work has been carried out and is worth recording.

Plasmid RP4 and RP1 which are broad host range plasmids for Gram negative bacteria have been transferred to T. neapolitanus, which is a non-acidophilic chemolithotroph (Kulpa et al., 1983). Carbenicillin resistance was used as the selected marker along with growth on minerals, however the donor was not counter selected and could have been maintained by crossfeeding. The same plasmids were transferred to T. versutus (which is non-acidophilic and organotrophic). In the recipients, neomycin, and

tetracycline resistance, were expressed, but not carbenicillin resistance (Plasota et al., 1984).

Davidson and Summers (1983) reported the transfer of several conjugative plasmids to T. intermedius, T. neapolitanus, T. perometabolis, or T. acidophilis. However, only T. novellus once having acquired the plasmids, could transfer them to all of the remaining Thiobacilli listed. Only T. acidophilis could not transfer plasmids back to E. coli once it had acquired the plasmids through conjugation with T. novellus. T. acidophilis was found to harbor three plasmids of its own (without known markers) while the only other strain with plasmids was T. intermedius which had one plasmid. This report is of considerable interest because of the possibility that T. acidophilus could serve as an intermediate host for transfer to T. ferrooxidans

Yankofsky et al. (1983) examined transformation in T. thioparus, T. neapolitanus, T. versutus and Thiobacillus strain Y. T. thioparus is evolutionarily the closest to T. ferrooxidans after T. thiooxidans (Lane et al., 1985). Yankofsky et al. (1983) reported a number of mutants of T. thioparus including streptomycin and naladixic acid resistant mutants and leucine, proline and tryptophan auxotrophs. Transformation of the mutants to prototrophy and transformation of the naladixic markers were reported. T. thioparus could be transformed with DNA from the wild type strain and with DNA from T. neapolitanus and from strain Y. However, the transformation frequencies were quite low.

Finally, there have been remarkably few reports of bacteriophage which infect Thiobacilli. Johnson et al., (1973) reported a phage for T. novellus.

No tests on other species of Thiobacillus were made and our search found no other reports of phage in this genus. The lack of phage seems unusual and might be an area for future investigations.

G. SUMMARY AND PROSPECTS FOR GENETIC ENGINEERING

The genetics of T. ferrooxidans are in a very primitive state, although what is presently known is encouraging with regard to genetic manipulation. There are two essential components to genetic engineering: (1) the methodology for transfer to and maintenance of DNA in T. ferrooxidans, and (2) an understanding of the signals which control expression of genes in T. ferrooxidans. These two components are linked experimentally. Both must be available for successful genetic engineering in T. ferrooxidans.

Virtually nothing is known about transfer of DNA into T. ferrooxidans. The reports of transformation studies either use other Thiobacilli or report negative results. Phylogeny studies have indicated that T. ferrooxidans is quite unrelated to other Thiobacilli, except for T. thiooxidans. Thus, studies in the heterotrophic Thiobacilli, like T. acidophilus, may be of little relevance. On the other hand, successful transformation studies require a test gene that is expressed in the recipient cell and such a gene is unavailable at present. In addition, there are new methods of transformation (such as electroporation) which have not been used. We consider this field of T. ferrooxidans research effectively untried at present.

In contrast, a considerable amount is known which has relevance to maintenance of genes in T. ferrooxidans, as several plasmids have been characterized. The fact that these plasmids are mobilized by other Gram negative conjugative plasmids is an encouraging sign, as it opens up the possibility of using well defined genetic systems. The results of Davidson and Summers (1983) on plasmid transfer between Thiobacillus species bear close scrutiny. Finally, the environment of T. ferrooxidans may well preclude the existence of bacteriophage, however such a situation would be unusual and given the potential value of a transduction vector, further work looking for phage which infect T. ferrooxidans should be done.

Since the ultimate goal of genetic engineering is to express genes in the host organism, information about the DNA and RNA sequences used to regulate gene expression in T. ferrooxidans is essential. The fact that at least the glutamine synthetase gene from T. ferrooxidans appears to function in E. coli, suggests that the regulatory signals in T. ferrooxidans might be similar to those in other Gram negative bacteria. Further work to examine other T. ferrooxidans genes by complementation of E. coli or other bacterial hosts is important to try to generalize the glutamine synthetase findings. However, ultimately the expression of genes in T. ferrooxidans itself will be required. One important approach to T. ferrooxidans gene expression is the generation of mutants. The search for mutants requires methods for growth on solid media, testing of growth conditions to find conditional mutants and testing of mutagens. An alternative method for studying T. ferrooxidans genetic regulation might be to examine mutants generated in T. ferrooxidans genes which can be shown to function in another host. For example, mutants

of T. ferrooxidans lacking glutamine synthetase activity could be used as a source of DNA for cloning. After recovery of the clone by hybridization methods, using the wildtype clone, changes in the DNA structure of the mutant gene could be examined. Finally, there are biochemical tests, such as S1 mapping to determine initiation sites of RNA synthesis which can be done with any gene cloned from T. ferrooxidans.

Although the information on T. ferrooxidans genetics and regulation is quite limited, the review of the literature suggests many of the features of T. ferrooxidans genetics and gene expression will be like those of other Gram negative bacteria. The major caveat is the question of genome instability raised by Holmes and Yates (personal communication). While, at the present time, the critical tools and information for genetic engineering in Thiobacillus are lacking, it seems likely that research into the basic genetics of T. ferrooxidans would lead to the development of those tools.

PLAN FOR GENETIC ENGINEERING IN THIOBACILLUS FERROOXIDANS

Our review of the literature suggests that at this time neither the genetic techniques nor biochemical background information is available to indicate potential genes which might be cloned or the procedures to be followed to improve leaching properties of T. ferrooxidans. Thus, on the whole, the priority in T. ferrooxidans research should be to better understand the physiology and the genetics of the organism. At the end of each review section we indicated areas which might be amenable to further study with the goal of genetic engineering. Below is a set of more specific research goals which should be followed.

A. The research described below should be carried out on pure strains, clearly devoid of acidophilic contaminants. We would suggest techniques such as those devised by Holmes (Yates and Holmes, 1985; 1986) using DNA probes, be widely applied before using cultures to carry out physiological studies. Furthermore, it would be advisable if most of the research were carried out on one particular strain of T. ferrooxidans. While it is not critical which strain is chosen, we would favor the ATCC strain used by Holmes for his genetic work.

B. While it is clear that T. ferrooxidans is critical in bioleaching, we would suggest that studies on defined mixtures of bacteria may be very useful for optimizing microbial leaching. These studies are beyond the scope of this report, but deserve mention.

C. The mechanism of metal sulfide oxidation by T. ferrooxidans has received a considerable amount of study. There remain several outstanding problems concerning the mechanism of oxidation of both iron and sulfur. A detailed understanding of these reactions is certainly worthwhile if they are to be the target of genetic manipulation. In particular, the role and the organization of the outer membrane; the nature of the sulfide oxidizing system and the mechanism for establishing and maintaining the membrane potential should be investigated. Many of the unresolved questions about metal sulfide oxidation relate directly to the organization of the components which carry out the oxidation reactions and how the metal sulfides are solubilized and transported to the site of enzymatic activity. The underlying principle of these studies should be to identify the rate limiting steps in the oxidation of metal sulfides by T. ferrooxidans.

D. A second factor in the efficiency of T. ferrooxidans is the nature of the adherence properties of the organism. The characteristics which lead to specificity in adherence are likely to be unique and deserve attention. In addition, further study is needed to address the basic question of whether adherence is required for maximum oxidation.

E. In contrast, the likelihood of creating psychrophilic strains through genetic engineering seems remote. There is not enough basic information on psychrophily in any system and what little information exists indicates that there are probably many genes involved. The most reasonable method for creating psychrophilic strains of T. ferrooxidans continues to be long term selection.

F. The potential exists to introduce genes for heavy metal resistance into T. ferrooxidans. For several of the metals of interest, there may exist gene systems in other bacteria. The key to success would be the ability to transfer and express the drug resistance genes in T. ferrooxidans. Some of the resistance mechanisms may function in the periplasmic space, so the transfer to T. ferrooxidans would require that the genes be modified so that their products could operate at low pH.

G. Considerable effort needs to be directed towards the molecular genetics of T. ferrooxidans. Research in each of the areas below could proceed relatively independently, however all will be needed to easily genetically manipulate T. ferrooxidans.

1. A mechanism for transferring genes into T. ferrooxidans is required. The search for bacteriophage would be a useful starting point. Studies on the molecular nature of the outer membrane and the production and regeneration of protoplasts might contribute substantially to the ability to transform the organism.

2. As mentioned earlier, the study of the molecular genetics of an organism requires genetic markers, that is mutants. Techniques for selection of mutants should be examined including sensitivity to mutagens and growth under conditions allowing selection of mutants (primarily growth on solid media).

3. Studies on the nature of cloned T. ferrooxidans genes and their expression in heterologous hosts should be supported. This work should include DNA sequence determination and should aim to define regions which are required for gene expression. Correlations with the points of initiation of RNA synthesis in the heterologous and homologous hosts should be made.

4. Additional work on the genome structure of T. ferrooxidans is warranted especially in the light of the work of Yates and Holmes. The potential for genetic instability particularly during changes in growth conditions needs careful examination.

5. While it is likely that the plasmids already identified could serve as cloning vehicles, continued search for plasmids particularly those associated with identifiable genetic traits would be useful.

H. While a discussion of environmental risks of genetic engineering in T. ferrooxidans is beyond the scope of this report, it is of sufficient importance that we feel it should be examined. It is likely that genetic engineering in T. ferrooxidans will proceed rather slowly for several years. During this time a thorough study of the possible effects of creating an "improved" T. ferrooxidans would be a high priority.

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APPENDIX A

STATEMENT FROM DRS. SPIEGLEMAN, BELL AND McBRIDE ON THE USE OF *THIOBACILLUS FERROOXIDANS* FOR MINING OF URANIUM

While we believe that the majority of research on *T. ferrooxidans* has been and will continue to be carried out for the general betterment of humankind, it would be a serious breach of our moral and scientific responsibilities to not include a statement on the use of *T. ferrooxidans* in the production of uranium for thermonuclear weapons.

We believe that thermonuclear weapons pose an urgent threat to humankind as a whole and to the security of Canada in particular, regardless of which nations possess these weapons. Canadian policy of rejecting the development of a nuclear arsenal has been a beacon of reason in the ever increasing shadow of the arms build up. As a means of reaffirming and strengthening both Canadian policy and the potential for disarmament, we urge that the Canadian government not sell or trade any fissile material, for example uranium recovered by bioleaching, whatsoever to any nation which currently possesses nuclear weapons, or which not having such weapons has not signed the Nonproliferation Treaty.

APPENDIX B

GLOSSARY

5S RNA sequence homology The extent to which the sequence of nucleotides in the 5S RNAs from two organism matches. 5S RNA (which is found in the ribosome in all cells) has been shown to be relatively conserved. The homology between 5S RNAs of different organisms can be used to measure the relatedness between organisms.

Adhesin A bacterial cell surface component which mediates binding of the cell to the substratum.

Antibiotic A substance, produced by one organism, which inhibits or kills another organism.

Amphiphillic A molecule having an uncharged or non-polar domain and a charged or polar domain e.g. lipopolysaccharide.

ATP-ase a complex of enzymes associated with the cytoplasmic membrane responsible for the synthesis of ATP. F_1, is the globular headpiece of this complex which bears the catalytic site. Fo spans the cytoplasmic membrane and provides a passage for protons to the F_1 headpiece.

Auxotroph/prototroph An auxotroph is a variant strain of an organism which requires a nutrient not required by its parental strain. The parental strain is termed a prototroph for that nutrient.

Bacteriophage A virus which only infects bacteria.

Capsule A layer found on the external surface of many bacterial cells; it is usually a carbohydrate polymer.

Cardinal The minimum, maximum and optimum values of an environmental parameter for growth.

Chemoautotroph A bacterium which gets its energy from the oxidation of inorganic molecules and its carbon by fixing CO_2.

Chemolithoheterotrophs See chemotrophy.

Chemolithotrophs See chemotrophy.

Chemotrophy A process used by cells to gain energy from the oxidation of an organic or inorganic chemical. Chemolithotrophs use only inorganic chemicals and usually get their carbon by assimilating CO_2. Chemolithoheterotrophs use inorganic chemicals to produce energy but get their carbon from organics.

Chimeric plasmids Plasmids containing DNA from two or more independent sources.

Competence A state of a cell when it has the ability to take up DNA molecules.

Conjugation The transfer of DNA from one cell to another. During transfer the donor cell and the recipient cell have to be juxtaposed.

Copy number The number of plasmids with the same structure and function in a given cell.

$\Delta\phi$ See electrochemical potential gradient.

$\Delta G^{0'}$ The standard free energy change of a chemical reaction when the initial concentration of each component is 1.0 M, the pH is 7.0 and the temperature is 25°C.

Diauxie Biphasic growth of a bacterial culture. In the first phase the most easily degraded substrate is utilized. When this is exhausted the second substrate is utilized.

DNA ligase A enzyme which joins the ends of pieces of DNA together by forming a covalent bond. It is commonly used *in vitro* to join DNA fragments cut by restriction endonucleases to plasmid DNA.

Electrochemical potential gradient The electric potential difference across the cytoplasmic membrane due to the accumulation of cations on the inner side of the membrane. $\Delta\phi$ is a measure of this potential in volts with the extracellular potential arbitrarily set at zero.

Electroporation A method for introducing DNA into cells under an electric field.

F_1Fo See ATP-ase.

Facultative chemolithotrophy A mode of metabolism where the bacterium is able to switch between chemolithotrophic growth or chemoheterotrophic growth (i.e. a mixotroph).

Fimbriae Hair-like structures of defined width and length protruding from the bacterial surface, composed of protein.

Fibrillae Hair-like structures of defined length and variable width protruding from the bacterial surface, composed of protein.

Gene A DNA sequence which encodes a functional protein, or RNA.

Gene dosage The number of genes with the same structure in a given cell.

Genome The molecule comprising the genetic information of an organism. For bacteria the genome is the chromosome.

Glycocalyx A carbohydrate polymer located on the external surface of a cell.

H^+/P ratio The number of protons passing through the F_1-Fo complex per molecule of ATP synthesized.

Heterotroph A bacterium which gets its carbon from the degradation and assimilation of organic molecules.

Km The Michaelis Menten constant. The concentration of substrate which allows the enzymic reaction to proceed at half its maximal rate.

Ks The concentration of a limiting substrate that will allow the cells to grow at 1/2 μ max.

LPS (Lipopolysaccharide) A constituent of the bacterial cell surface composed of lipid linked to a complex polysaccharide. The polar carbohydrate is oriented towards the aqueous environment.

Lysogenic A type of bacteriophage which can, instead of lysing a host cell, direct the integration of its DNA into the bacterial chromosome. The inserted bacteriophage DNA is then replicated along with the bacterial DNA.

Molecular cloning The introduction of a particular DNA sequence into a vector molecule to produce multiple copies of the sequence.

μ max The maximum specific growth rate. This is a measure of how fast the cells can grow under a given set of conditions.

Mixotroph A bacterium which is able to switch from chemoautotrophic growth to chemoheterotrophic growth and vice versa.

mRNA (messenger RNA) A form of RNA which is used to direct synthesis of proteins by specifying the order of addition of amino acids.

Mutation A change in the sequence of nucleotides in the genome of an organism.

NAD^+/NADH Oxidized and reduced nicotinamide adenine dinucleotide. An organic molecule used as a carrier of reducing equivalents (protons) to the cytochromes.

Nucleotides Purine or pyrimidine bases bonded to a deoxyribose-phosphate or ribose-phosphate. Phosphate-ribose bonds between nucleotides make up the backbone of a DNA or RNA chain. In DNA the bases can be either adenine (A), guanine (G), cytosine (C) or thymidine (T). In RNA uracil substitutes for thymidine. It is the sequence of nucleotides in the DNA which determines the sequence of amino acids in a gene product.

Obligate chemolithotrophy A mode of metabolism where the bacterium has no means of growth other than chemolithotrophy.

Origin of replication The site on a DNA molecule where DNA replication is initiated.

Peptidoglycan Carbohydrate polymers linked by peptides which form a rigid protective layer surrounding the cell cytoplasmic membrane.

Pili Similar to fimbriae.

Plasmid DNA A circular double stranded piece of DNA which is not physically linked to the chromosome and capable of independent regulation. Plasmids vary in size from 1000 nucleotides (1 kb) to over 300 kb and vary in the number per cell (copy number) from 1-100 per chromosome. Plasmids are one of the most popular vectors for molecular cloning.

Poly U translation The *in vitro* synthesis of a protein from a mRNA which is composed solely of uracil (U). The artificial protein formed consists solely of phenylalanine.

Porin A bacterial outer membrane protein which forms pores facilitating transport of small molecules.

P/O ratio The number of ATP molecules synthesized per molecule of NADH oxidized.

Protein Proteins are composed of a chain of amino acids. The order of the amino acids for each protein is determined by the nucleotide sequence on one segment of the DNA.

Protonophores See uncouplers.

Protoplast A bacterial cell which has had all its cell wall removed usually by the action of lytic enzymes. Protoplasts are able to fuse together to make hybrid cells.

Prototroph See auxotroph.

Psychrophile A bacterium which can grow at 0°C and has an optimum temperature for growth < 15°C.

Q_{10} A quotient computing the change in the rate of a reaction for every 10°C change in temperature.

QO_2 The uptake of oxygen measured per unit cell dry wt/hour.

Replication The duplication of chromosomal or plasmid DNA.

Recombinant DNA A DNA molecule containing pieces which are not normally contiguous, and often derived from different organisms, which are covalently linked to each other by *in vitro* techniques.

Replicon A piece of DNA carrying sufficient information to allow it to be replicated in a given cell.

Restriction endonucleases Enzymes which recognize a specific sequence of nucleotides in double stranded DNA and cleave the DNA at the recognized site, generating pieces with either blunt ends or staggered ends.

Restriction endonuclease fragment length polymorphisms Differences between organisms in the pattern of fragments of a particular segment of DNA which are generated by treatment with a restriction endonuclease. These differences result from mutations which alter sites recognized by the restriction endonuclease.

RNA Ribonucleic acid having ribose instead of deoxyribose in the backbone and uracil instead of thymidine as one of the bases. RNA is found in ribosomes (rRNA); as carriers of amino acids for protein synthesis (tRNA) and as the template for protein synthesis (mRNA).

Sequence of a gene The order of nucleotides in a gene. The average gene is about 1000 nucleotides long.

Thermophiles Bacteria which can only grow at temperatures greater than 45-50°C.

Transduction vector Transduction is the transfer of genes between bacteria by one of several mechanisms which use bacteriophage. A transduction vector is a bacteriophage which can carry out transduction.

Transformation A mechanism of transfer of bacterial DNA which involves the uptake of DNA by a competent cell. After the DNA has entered the cell it may recombine with the host chromosome or replicate independently as a plasmid.

tRNA See RNA.

Uncouplers Agents which render the cytoplasmic membrane leaky to protons. A proton gradient can not therefore be established so that proton extrusion is uncoupled from ATP synthesis. Protonophores are a specific form of uncouplers which transport protons into the cell.